A study of the regulation of Trib family members expression in normal and malignant haematopoiesis

Thesis for the degree of Doctor of Philosophy

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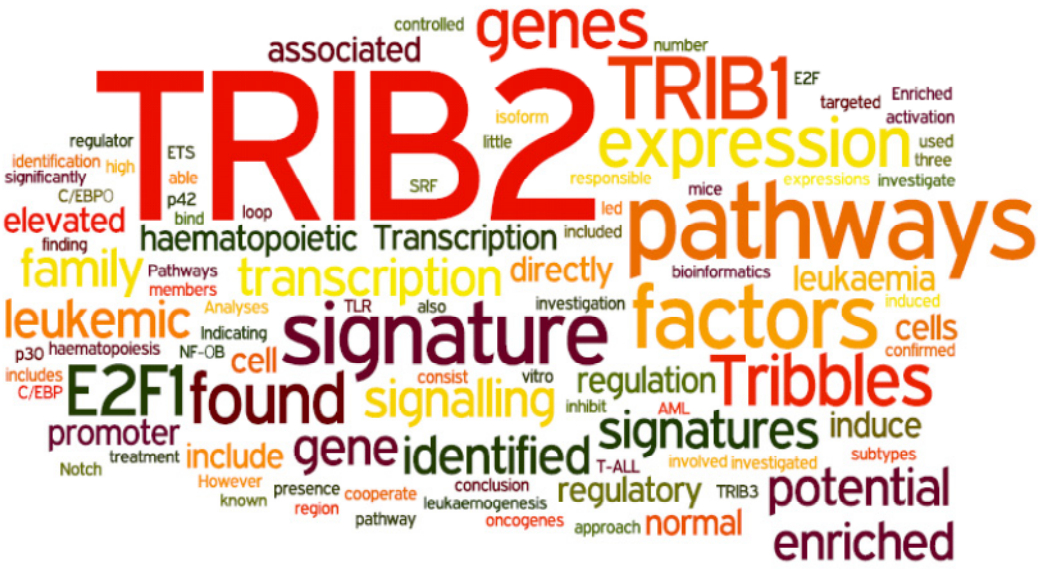
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This thesis is dedicated to my Uncle, Tony Mulrennan, who passed away in September 2013

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Declaration

This thesis has not been previously submitted, in part or in whole, to this or any other University for any degree and is, unless otherwise stated, the original work of the author.

Date: _____

Signed: _____

Maura Hannon

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ABSTRACT

The Tribbles family of genes consist of three members; TRIB1, TRIB2 and TRIB3. Trib1 and Trib2 have been identified as oncogenes that can induce AML in mice. However little is known about how the expressions of the Tribbles family genes are controlled in the cell during haematopoiesis or leukaemogenesis.

To investigate the Tribbles genes in leukaemia a bioinformatics approach was used. TRIB2 expression was found to be elevated in T-ALL and ALL with t(1;19). TRIB1 was found not to be significantly elevated in any leukaemic subtypes. Analyses of the TRIB1 and TRIB2 gene signatures in both leukaemic and normal haematopoietic cells identified pathways and transcription factors associated with these signatures. Pathways enriched for the TRIB1 signature included TLR signalling pathways and NF-κB pathways. Transcription factors enriched for this signature include C/EBP and SRF. Enriched for the TRIB2 signature includes T cell signalling pathways and Notch signalling pathways. Transcription factors enriched for this signature include E2F and ETS.

Further investigation *in vitro* confirmed the finding that E2F1 was as a potential regulator of TRIB2 expression. E2F1 is able to directly bind to the TRIB2 promoter region and induce TRIB2 expression. C/EBPα p42 was found to inhibit E2F1 and the p30 isoform was found to cooperate with E2F1 induced activation of the TRIB2 promoter. Indicating the potential presence of a regulatory loop involved in the regulation of the TRIB2 gene.

In conclusion we have investigated the Tribbles gene signatures in both normal haematopoietic and leukaemic cells. This has led to the identification of a number of

pathways and transcription factors associated with these genes. We have also identified a family of transcription factors directly responsible for the regulation of TRIB2 expression. This regulatory pathway has the potential to be targeted in the treatment of leukaemia with a high TRIB2 signature.

Abbreviations

Abbreviations	Meaning
ACC	Acetyl-CoA Carboxylase
AcLDL	acetylated low-density lipoprotein
ACOX	Peroxisomal Acyl-Coenzyme A Oxidase
ALCL	Anaplastic Large Cell Lymphoma
ALL	Acute Lymphoblastic Leukaemia
AML	Acute Myeloid Leukaemia
ANKRD	Ankyrin Repeat Domain-Containing Protein
ANOVA	Analysis of Variance
APL	Acute Promyelocytic Leukaemia
ATF	Activating Transcription Factor
ATRA	All-Trans Retinoic Acid
BAX	Bcl-2-Associated X
BCELL	B Cells
BCL-2	B-Cell Lymphoma 2
BMMCs	Bone Marrow-Derived Mast Cells
bp	Base Pairs
BSA	Bovine Serum Albumin
C/EBP	CCAAT enhancer binding protein
CAM	Cell Adhesion Molecules
cAMP	Cyclic Adenosine Monophosphate
CASP	Caspase
CD	Cluster of Differentiation
CDC	Cell Division Cycle
cDNA	Complementary DNA
CFU	Colony Forming Unit
CHOP	C/EBP Homologous Protein
CLL	Chronic Lymphoblastic Leukaemia
CLP	Common Lymphoid Progenitor
CMP	Common Myeloid Progenitor
COP1	Coat Protein 1
CPT	Carnitine Palmitoyltransferase
CRC	Colorectal Cancer
CREB	cAMP response element-binding protein
CTGF	Connective Tissue Growth Factor
CTNNB1	β-Catenin
CUL	Cullin
DDIT3	DNA Damage-Inducible Transcript 3
DHMEQ	Dehydroxymethyl-Epoxyquinomicin
DMEM	Dulbecco’s Modified Eagle’s Medium
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol

Early Mye	Early Myeloid Cells
Early Mye + T/B-cell +GRAN	Early Myeloid, the T and B Cells and the Granulocytes
EBS	Ets Binding Sites
ECL	Enhanced Chemiluminescence
ECT2	Epithelial Cell Transforming 2
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EGR-1	Early Growth Response Protein 1
EphB6	Ephrin Type-B Receptor 6
ER	Endoplasmic Reticulum
ERK	Extracellular Signal-Regulated Kinase
ES	Enrichment Score
ETS	E-Twenty Six
FABP10a	Fatty Acid Binding Protein 10a
FBS	Foetal Bovine Serum
FBXW7	F-box/WD Repeat-Containing Protein 7
FDR	False Discovery Rate
FOX	Forkhead Box Proteins
Fz	Frizzled
G-CSF	Granulocyte Colony-Stimulating Factor
GEO	Gene Expression Omnibus
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
GMP	Granulocyte-Monocyte Progenitor
GRAN	Granulocytes
GSEA	Gene Set Enrichment Analysis
hASMC	Human Aortic Smooth Muscle Cells
HDAC	Histone Deacetylases
HDACI	Histone Deacetylases Inhibitors
HDL	High-Density Lipoprotein
HEK293T	Human Embryonic Kidney 293T cells
HEL	Human Erythroleukemia Cell Line
Hela cells	Henrietta Lacks Cells
Hes1	Hairy and Enhancer of Split-1
HHcy	Hyperhomocysteinemia
HOX	Homeobox Genes
HRP	Horseradish Peroxidase
HSC	Haematopoietic Stem Cell
HuR protein	Hu-antigen R protein
ICNX	Intracellular Notch1
IFN-γ	Interferon Gamma
IL	Interleukin
IMDM	Iscove Modified Dulbecco Medium
IPs	Immunoprecipitations

IRS-1	insulin receptor substrate 1
IT	intermediate-term
JAG1	Jagged 1
JAK2	Janus Kinase 2
JNK	c-Jun N-terminal Kinase
kb	Kilobase
KO	Knockout
Late Ery	Late Erythrocytes
Late MYE	Late Myeloid Cells
LB	Luria-Bertani
LDL	Low-Density Lipoprotein
LFLS	Li-Fraumeni-like Syndrome
LMPP	Lymphoid-Primed Multipotent Progenitor
LPS	Lipopolysaccharides
LT	Long-Term
MAPK	Mitogen-Activated Protein Kinase
MAPKK	MAPK Kinase
MAPKKK	MAPKK Kinase
MDS	Myelodysplastic Syndromes
MEF	Murine Embryonic Fibroblast
MEP	Megakaryocyte-Erythrocyte Progenitor
MHC Class 1 Receptors	Major Histocompatibility Complex Class 1 Receptors
MILE Study	Microarray Innovations in Leukaemia Study
miR	micro-RNA
MLPs	Immature Lymphoid Progenitors
MPP	Multipotent Progenitor
mRNA	Messenger RNA
MSigDB	Molecular Signalling Database
MTOR	Mammalian Target of Rapamycin
Mut.	Mutant
NCRI	National Cancer Registry Ireland
NES	Negative Enrichment Score
NF-κB	Nuclear Factor-kappaB
NGF	Nerve Growth Factor
NK cells	Natural Killer Cells
NKA	Activated NK Cells
NKT	Natural Killer T cells
NM	Neutrophilic Metamyelocyte
NOM p-val	Nominal p-values
NSCLC	Non-Small Cell Lung Cancer
NuP98	Nucleoporin 98kDa
OTSCC	Oral Tongue Squamous Cell Carcinoma
PBS	Phosphate Buffered Saline
PBX1	Pre-B-cell Leukemia Homeobox 1

PCR	Polymerase Chain Reaction
PERK	PRKR-Like Endoplasmic Reticulum Kinase
PGC-1	Peroxisome Proliferator-Activated-γ Coactivator-1
Ph	Philadelphia Chromosome
PI3K	Phosphatidylinositol-3-Kinase
PKB	Protein Kinase B
PMA	Phorbol Myristate Acetate
PNT domain	Pointed domain
PPAR	Peroxisome Proliferator-Activated Receptor
pRB	Retinoblastoma
qPCR	Quantitative Polymerase Chain Reaction
RAR	Retinoic Acid Receptor
RARE	Retinoic Acid-Responsive Element
RCF	Relative Centrifugal Force
RIPA	Radioimmunoprecipitation Assay Buffer
RNase	Ribonuclease
RPM	Revolutions Per Minute
RPMI 1640	Roswell Park Memorial Institute medium
RT-PCR	Reverse Transcription Polymerase Chain Reaction
Scd1	stearoyl-CoA Desaturase 1
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SKP1	S-Phase Kinase-Associated Protein 1
SNPs	Single-Nucleotide Polymorphisms
SRE	Serum Response Elements
SRF	Serum Response Factors
SSC Mapping	Connectivity Mapping
ST	Short-Term
STAT	Signal Transducers and Activators of Transcription
SVF	Stroma Vascular Fraction
T/B cell	T and B cells
T2P	Trib2 Promoter
T2P DM	T2P Double Mutant
T-ALL	T-Acute Lymphoblastic Leukaemia
Tcell + NK	T cells and NK cells
TEAD	Transcriptional Enhancer Factor TEF
TESS	Transcription Element Search System
TFT	Transcription Factor Target
TG	Triglyceride
TGF-β	Transforming Growth Factor β
THC	Δ^9-tetrahydrocannabinol
TLR	Toll-like receptor
TNF-α	Tumor Necrosis Factor alpha

TP53	Tumor Protein p53
TRAIL	TNF-Related Apoptosis-Inducing Ligand
TRD@	T Cell Receptor Delta Locus
Treg Cell	Regulatory T Cell
UDP	Uniparental Disomies
UV	Ultraviolet
VDRE	Vitamin D-Responsive Element
VLDL	Very-Low-Density Lipoprotein
WAT	White Adipose Tissue
YAP	Yes-Associated Protein

Chapter 1

Introduction

1.1 The Tribble Family

The Tribble gene was first identified in Drosophila as a gene involved in the control of cellular proliferation and migration. Subsequently three Tribble homologues have been identified in mammals, TRIB1, TRIB2 and TRIB3. The Tribble proteins are considered to be signal modulators, involved in the control and transduction of extra- and intracellular signals in the cell. Correct control of these signalling pathways by the Tribble proteins via interaction with various kinases, transcription factors and ubiquitin ligases allows for the appropriate coordination of cell signalling and cellular responses such as proliferation, differentiation and apoptosis (Dobens and Bouyain, 2012). Dysregulation of these cellular response pathways can lead to a vast array of disease states including cancer, inflammatory disorders and diabetes mellitus (Yokoyama and Nakamura, 2011; E Dugast et al., 2013; K. L. Liang et al., 2013), all of which have been linked to aberrant Tribble function or expression in the cell.

While not found in fungi, plants or prokaryotes a Tribble-like sequence can be found in the unicellular Monosiga ovata; this represents the earliest point in the evolutionary tree where a Tribble-like sequence can be found. This indicates that the Tribble protein is ancient. As the Tribble protein evolved it began to segregate, from a single gene in invertebrates, to the three Tribble homologies found in mammals (Trib1, Trib2 and Trib3). In the majority of cases the Tribble genes are comprised of three exons in vertebrates, regardless of the subfamily classification of the protein encoded (Hegedus et al., 2006). Bioinformatic analysis suggests that Tribble proteins contain an evolutionary conserved central kinase-like domain and short flanking N- and C- terminal sequences (figure 1.1). The evolutionary conservation of the kinase-

like domain is asymmetric as conservation is more profound in the C-terminal section of the domain which also includes conserved Coat Protein 1 (COP1) and MEK1 binding sites (Hegedus et al., 2006; Dobens and Bouyain, 2012) (figure 1.1).

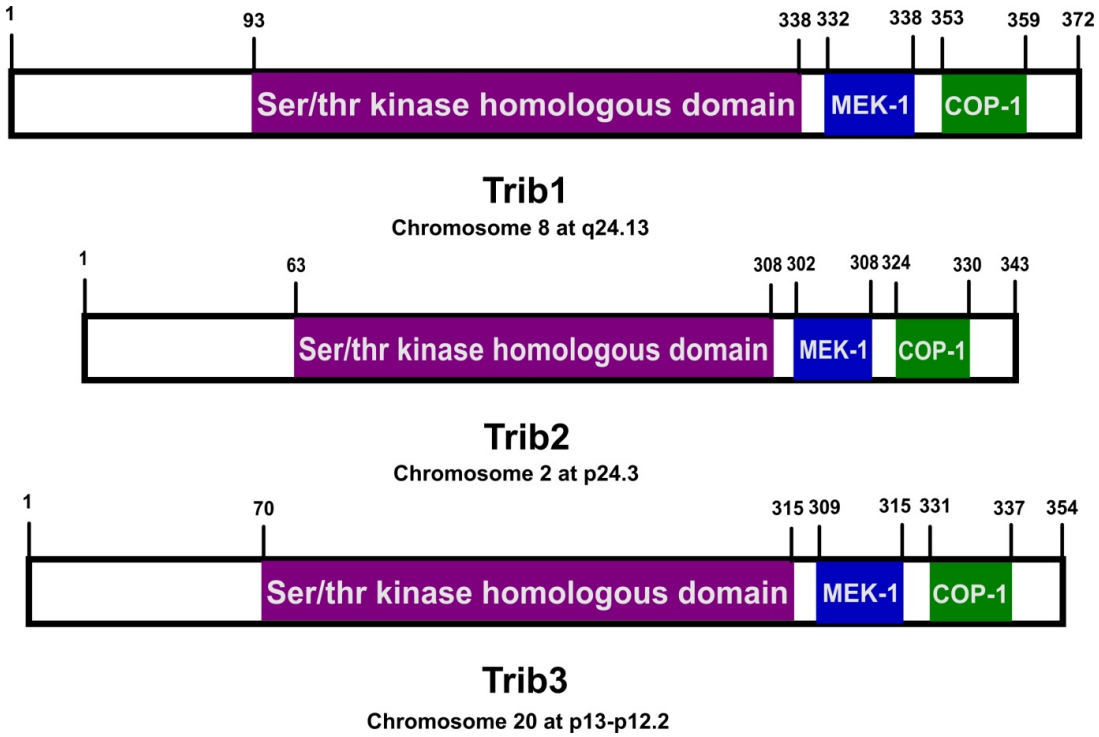

Figure 1.1: the structure of the three mammalian Tribble genes; Trib1, Trib2 and Trib3. The three Tribble genes all contain a conserved central ser/thr kinase homologous domain (purple), a mek-1 binding domain (blue) and a cop-1 binding domain (green). location of each of the Tribble genes in the human chromosome is also indicated under the name of each gene (Hegedus et al., 2006; Dobens and Bouyain, 2012).

The Tribble proteins have been identified as three of the 48 human protein kinases that have been classified as pseudokinases, which make up approximately 10% the kinome. A pseudokinase is a kinase-like protein that lacks one or more of the conserved amino acids that characteristically make up the kinase domain of a protein. In the Tribble proteins case they lack the Asp-Phe-Gly (DFG) motif found in

subdomain VII of the kinase domain. As these conserved amino acids are necessary for kinase activity these psedokinases are predicted to be catalytically inactive (Manning et al., 2002; Boudeau et al., 2006). As the Tribble proteins are predicted to lack the kinase catalytic activity these proteins are thought to have other non-catalytic functions in the cell. It has been suggested that they and other psudokinase proteins may act as scaffold proteins participating in the assembly of multi-protein complexes as these proteins contain protein-protein interaction domains as well as the kinase-like domain (Boudeau et al., 2006). It has even been suggested that the Tribble proteins may act as decoy kinases impinging on kinase activity by interfering with protein binding between a kinase and its substrate (Lohan and Keeshan, 2013).

The Tribble genes encode mRNA with shorter than average half lives. On average the median half life for mRNA in murine cells is approximately 7.1 hours. However Trib1 mRNA, like the mRNA of less than 100 other genes, has been shown to have a half life of less than one hour (Sharova et al., 2009). The same study determined that Trib2 has an average half-life of 2.6 hours and Trib3 has a half-life of approximately 2.8 hours.

Trib3 is highly expressed in the human and murine liver and in the murine small intestine (Bowers et al., 2003; Okamoto et al., 2007). While Trib1 is highly expressed in the murine liver and heart, Trib2 expression is high in the murine thymus as well as in the brain, heart, kidneys and lungs (Okamoto et al., 2007).

1.2 Function of Tribble in the Cell

The Tribble proteins seem to play key roles in a number of diverse cellular functions including haematopoiesis, the immune response and apoptosis. These proteins can

regulate these functions through their involvement in diverse cellular processes such as cell signalling, protein activation and protein degradation. The Tribble are known to be involved in the following pathways.

1.2.1 Adaptors in the MAPK Signalling Pathway

One of the first cellular signalling pathways linked to Tribble expression was the mitogen-activated protein kinase pathway or MAPK pathway. This pathway is activated by a phosphorylation cascade in the cell and has been shown to regulate a large number of cellular processes including cellular differentiation, proliferation and death (Pearson et al., 2001). In 2004 Kiss-Toth et al. reported that the Tribble proteins possess the ability to control MAPK cascades. They discovered that the Tribble possessed the ability to bind to MAPK kinase (MAPKK) and regulate the activation of the MAPK pathway.

The MAPK signalling pathways are activated by a number of extracellular signals including growth factor and stress signals. Activation of the MAPK pathway results in a signal cascade consisting of three proteins. Initial signalling, by a growth factor for example, leads to the phosphorylation and subsequent activation of MAPKK kinase proteins (MAPKKKs) which in turn leads to the phosphorylation and activation of MAPK kinases (MAPKKs) leading to the phosphorylation/activation of a mitogen-activated protein kinase (MAPK). These three proteins make up a signalling module in the cascade and are brought together by scaffolding proteins which tether the proteins involved in the cascades into specific modules. Four distinct subfamilies of MAPKs have been identified in mammalian cells. These include the extracellular signal-regulated kinases (ERK1 and ERK2), c-Jun NH_2-terminal kinases (JNK1, 2 and 3), p38 MAPK (p38α. p38β, p38γ and p38δ) and the

most recently discovered ERK5. Different extracellular signals activate different MAPK subfamilies. For example ERK 1/2 are activated primarily by growth factors while the JNK kinases are activated by environmental stress. Many of the subfamilies of MAPK are activated by cytokines and cross-talk between the pathways can occur (Garrington and Johnson, 1999; Johnson and Lapadat, 2002; Nithianandarajah-Jones et al., 2012).

MAPKK were found to directly interact with the TRIB1 and TRIB3 proteins. Both TRIB1 and TRIB3 interact with MEK-1, which is involved in the ERK 1 and 2 signal transduction pathway (Roberts and Der, 2007). MKK-4 and MKK-7 are primarily associated with the JNK1 and 2 signalling pathway (Haeusgen et al., 2011). MKK-4 specifically interacted with TRIB1 while interaction with MKK-7 was specific to TRIB3. These interactions were found to stabilise the TRIB1 and 3 proteins and MEK-1 and MKK-7 were found to increase TRIB3 expression while MKK-4 increased TRIB1 expression. Interestingly MAPK activation by TRIB3 was found to be dose dependent. Low levels of TRIB3 activated the MAPK pathways, however increasing TRIB3 levels lead to an inhibition of MAPK signalling (Kiss-Toth et al., 2004). This indicates that the function of the Tribble proteins can vary depending on dose.

Functionally TRIB2 was found to repress monocyte activation by modulation of the MAPK pathway in the cell. By interacting the MEK1 and MKK7, but not MKK4, TRIB2 was able to suppress IL-8 production by the monocyte cell following inflammatory signal stimulation by low-density lipoprotein (LDL) and AcLDL. Knock-down of TRIB2 expression led to a significant increase in JNK and ERK activation and an increase in IL-8 production by the monocytes following stimulation of the cells (Eder et al., 2008b).

TRIB1 expression was also found to influence cellular processes by affecting the MAPK signalling pathway. Functionally TRIB1 was shown to control both the proliferation and the chemotaxis of smooth muscle cells, a hallmark of atherosclerosis, via the MAPK signalling cascade (Sung et al., 2007). Patients with ischemic heart disease showed an increase in TRIB1 (but not TRIB2) expression in human aortic smooth muscle cells (hASMC). TRIB1 was up-regulated in response to inflammatory stimulation of the hASMC cells by LPS. While increasing TRIB1 expression had a modest anti-proliferative effect on the cells, reducing TRIB1 levels resulted in a significant increase in the proliferation of hASMC. The JNK pathway, which suppressed hASMC proliferation, was in turn suppressed by TRIB1. The key to the regulation of the JNK pathway was found to be the direct interaction of TRIB1 and MKK4 which can activate JNK. The central kinase-like domain of TRIB1 was sufficient for this interaction to take place while the N-terminal region of the TRIB1 protein was critical for the nuclear localisation of the TRIB1/MKK4 complex (Sung et al., 2007).

In leukaemia Trib1 expression was also found to significantly enhance the activation of the MAPK signalling pathway by mediation of the phosphorylation of ERK. Previously murine Trib1 over-expression in Henrietta Lacks cells (Hela cells) was found to increase the phosphorylation of ERK upon Phorbol Myristate Acetate (PMA) stimulation (Jin et al., 2007). When Trib1 and Hoxa9/Meis1-induced leukemic cells were stimulated with IL-3 they also showed this enhanced phosphorylation of ERK compared to Hoxa9/Meis1-induced leukemic cells without Trib1. Enhanced phosphorylation of ERK was also found in primary bone marrow cells with Trib1 even after IL-3 withdrawal and Trib1 positive leukemic cells were found to be more resistant to apoptosis after IL-3 withdrawal (Jin et al., 2007).

The importance of the MEK1/ERK pathway in Trib1 leukaemogenesis was further highlighted by the discovery that that Trib1 linked the MEK1/ERK pathway in leukaemogenesis and that MEK1 binding was vital for the induction of leukaemia by Trib1 (Yokoyama et al., 2010). Trib1 was found to interact with MEK1 via a conserved septapeptide domain in the C-terminus of the Trib1 protein. This interaction was necessary for the phosphorylation of ERK by MEK1 in the cell and the septapeptide domain was found to be necessary for Trib1 induced leukaemogenesis and cooperation with Hoxa9 and Meis1.

1.2.2 Modulators of C/EBP Proteins Levels

The CCAAT enhancer binding proteins or C/EBPs are a family of highly conserved transcription factors involved in the regulation of a diverse range of cellular processes including differentiation, inflammation and proliferation. C/EBPs all contain a conserved basic-leucine zipper domain at the C-terminus that is involved in the dimerization and DNA (Deoxyribonucleic Acid) binding of these proteins. To date six members of the C/EBP family have been described; C/EBPα, C/EBPβ, C/EBPδ, C/EBPε, C/EBPγ and C/EBPζ (Ramji and Foka, 2002). C/EBPα has been identified as a key myeloid differentiation factor involved in both granulopoiesis and monopoiesis. Depletion of C/EBPα protein levels or mutation of the C/EBPα gene (but not the total abolition of C/EBPα expression) in the myeloid progenitor cell has been identified as a contributing factor to myeloid transformation resulting in a block in myeloid differentiation and a promotion of myeloid progenitor cell cycle progression (Zhang et al., 1997; Porse et al., 2001; Zhang et al., 2004; Heath et al., 2004; Porse et al., 2005; Kirstetter et al., 2008; Hasemann et al., 2008).

The first evidence that the Tribble proteins may play a role in the regulation of the C/EBPs was discovered when over-expression of the Drosophila trbl protein was found to block migration of border cells by binding to and promoting the proteosomal degradation of slbo, a fly C/EBP homologue (Rørth et al., 2000). The kinase domain of trbl was found to be required for this interaction with slbo which was also found to be necessary but not sufficient to suppress trbl expression (Masoner et al., 2013).

It was subsequently discovered that Trib2 could degrade the full length p42 isoform of the C/EBPα protein in mice. This degradation of the C/EBPα protein by over-expressed Trib2 lead to a block in myeloid differentiation in the haematopoietic compartment and the mice consequently developed acute myeloid leukaemia (AML) (Keeshan et al., 2006). Further investigation showed that Trib1 could also degrade C/EBPα and aberrant Trib1 expression also lead to the development of AML in mice (Jin et al., 2007; Dedhia et al., 2010). Trib3 was found to lack the ability to degrade C/EBPα and over-expression of Trib3 did not lead to leukaemia in mice (Dedhia et al., 2010).

In both the non-leukemic and leukemic cells of haematopoiesis the degradation of C/EBPα by Trib1 and Trib2 is mediated by a COP1 binding domain present in both these proteins. This COP1 domain is critical for the leukemic activity of both Trib1 and Trib2 as well as Trib1 mediated differentiation in haematopoietic cells (Keeshan et al., 2010; Yokoyama et al., 2010; Satoh et al., 2013). COP1 was also found to cooperate with Trib1 in the induction of murine AML (Yoshida et al., 2013). COP1 is a ubiquitin ligase and recent investigations have revealed that COP1 complexes with Trib1 in order to target C/EBPα for degradation in the cell (Yoshida et al., 2013). Though Trib3 also contains this conserved COP1 binding domain (figure 1.1)

(Dobens and Bouyain, 2012) it cannot degrade C/EBPα in the cell (Dedhia et al., 2010) despite the fact that it has been shown to interact with COP1 (Qi et al., 2006) suggesting that other interactions may also play a role in Trib1 and Trib2 mediated degradation of C/EBPα.

Trib1 has been found to link the degradation of C/EBPα and the MAPK signalling pathway in leukaemia. Trib1 over-expression enhances the activation of the MAPK signalling pathway in leukaemia (Jin et al., 2007; Yokoyama et al., 2010). It was discovered that Trib1 interacts with MEK1 via a conserved septapeptide domain which also contains the COP1 binding domain necessary for Trib1 mediated degradation of C/EBPα. Inhibition of MEK1 resulted in the loss in the ability of Trib1 to degrade C/EBPα in the cell thus linking these two pathways together via Trib1 in the leukemic cell (Yokoyama et al., 2010).

While over-expression of Trib1 causes leukaemia normal Trib1 expression was found to be critical for the regulation of myeloid differentiation in haematopoiesis. Similar CMP, CLP and GMP population sizes and comparable patterns of transcription factor expression in the GMP of wildtype and Trib-deficient mice indicates that Trib1 may act downstream of the GMP population. Trib1-deficient mice were found to lack tissue-resident M2-like macrophages (associated with responses to anti-inflammatory reactions and tumour progression) and eosinophils. These defects in myeloid differentiation were attributed to aberrant C/EBPα expression in the haematopoietic compartment (Satoh et al., 2013). Depletion of C/EBPα by TRIB2 has been implicated as a factor contributing to the oncogenic effects of TRIB2 in other solid cancers including lung and liver cancer (Grandinetti et al., 2011; J. Wang et al., 2013; P.-Y. Wang et al., 2013). In non-small cell lung

cancer cell lines the E3 ubiquitin ligase TRIM21 was necessary for TRIB2 mediated degradation of C/EBPα (Grandinetti et al., 2011).

The Tribble proteins have also been implicated in the regulation of the C/EBPβ isoform of the C/EBP proteins. Microarray analyses of wild-type versus TRIB1 deficient macrophages revealed that a subset of LPS-inducible genes, independent of the activation of the Nuclear Factor-kappaB (NF-κB) and the MAPK pathways, are dysregulated in the TRIB1-deficient cells (Yamamoto et al., 2007; Y.-H. Liu et al., 2013). This led to the identification of a protein-protein interaction between C/EBPβ and TRIB1. TRIB1 over-expression in 293T and RAW cells leads to a decrease in C/EBPβ protein levels, while TRIB1 deficient macrophages showed an increase in C/EBPβ protein and mRNA levels. TRIB1 has also been identified as a direct target of C/EBPβ in ALK+ Anaplastic Large Cell Lymphoma (ALCL). TRIB1 expression was decreased upon knock-down of C/EBPβ expression and C/EBPβ was found to bind to a regulatory region of the TRIB1 locus (Bonzheim et al., 2013). TRIB2 was also found to interact with and reduce the levels of the LAP protein isoform of C/EBPβ, but not the LIP protein isoform and C/ABPδ in 3T3-L1 cells (Naiki et al., 2007).

While both TRIB1 and TRIB2 can degrade members of the C/EBP family of proteins emerging evidence suggest that members of the C/EBP family can induce the expression of the Tribble genes. Both TRIB1 and TRIB2 expression is induced by C/EBPα in 293T cells (Gilby et al., 2010). Stimulation of the TRIB3 expression by fibrates (drugs that function by binding and activating peroxisome proliferator-activated receptor α (PPARα)) was found to occur via the induction and subsequent recruitment of CHOP and C/EBPβ to the TRIB3 promoter mediated by fibrates (Selim et al., 2007).

1.2.3 Inhibitors of AKT Activation

AKT (also known as Protein Kinase B (PKB)) is frequently found to be constitutively activated in many different types of human tumours. Modulated downstream of phosphatidylinositol-3-kinase (PI3K) in response to a number of different extracellular stimuli, such as growth factor and hormones, the serine/threonine protein kinase AKT is involved in many cellular processes including cell growth, proliferation, metabolism and survival. (Brazil and Hemmings, 2001; Nicholson and Anderson, 2002).

TRIB3 is a target of the PI3K pathway (Schwarzer et al., 2006) and both TRIB2 and TRIB3 have been found to interact with AKT *in vivo* and expression of both of these genes was found to inhibit phosphorylation of AKT thereby inhibiting AKT activation in the cell (Du et al., 2003; Ding et al., 2008; Xie et al., 2012). TRIB2 and TRIB3 have been shown to suppresses adipocyte differentiation by their ability to suppress AKT activation (Naiki et al., 2007). Inhibition of AKT activation was also found to induce the expression of TRIB1 in non-small-cell lung carcinoma (NSCLC) cells (C. Zhang et al., 2011).

One of the many extracellular signals that trigger the phospholipid-dependent kinase cascade culminates with the phosphorylation of the Ser-Thr kinase AKT is the binding of insulin to its receptor. Insulin signalling is responsible for the promotion of the uptake of glucose into the cell, particularly muscle cells, and the suppression of glucose production in the liver; resistance to insulin signalling is one of the hallmarks of type II diabetes (Brazil and Hemmings, 2001; Schinner et al., 2005). TRIB3 expression is induced in liver and muscle cells in response to insulin signalling leading to the inhibition of AKT signalling within the cell (Du et al., 2003;

Matsumoto et al., 2006; Ding et al., 2008; Du and Ding, 2009; Koh et al., 2013). In response to insulin signalling in the liver TRIB3 directly interacts with AKT inhibiting its phosphorylation, this prevents AKT from associating with the plasma membrane leading to an inhibition of the AKT cell signal (He et al., 2006).While TRIB3 can inhibit AKT, AKT can also inhibit TRIB3 expression by suppressing its promoter activity (Ding et al., 2008).

TRIB3 regulation of AKT activation is also observed in other cell types. Over-expression of TRIB3 in C2C12 muscle cells and in the skeletal muscle of mice results in a significant decrease in the phosphorylation of AKT and insulin receptor substrate 1 (IRS-1) (Koh et al., 2013), an upstream activator of the PI3K pathway important in the transduction of the insulin response signal in the cell (Esposito et al., 2001).

TRIB3 can also inhibit AKT activation in response to other extracellular signals. In rats, chronic ethanol consummation leads to an increase in the hepatic levels of Trib3 mRNA and protein. Chronic ethanol consummation was shown to inhibit hepatic AKT phosphoylation of via Trib3 leading to a decrees the AKT activity (He et al., 2006).

TRIB3 expression inhibits both endothelial and hepatocyte proliferation in response to homocysteine signalling through inhibition of AKT. This leads to cell cycle arrest inhibiting the proliferation of these two cell types (Zou et al., 2011; Yu et al., 2013). Hyperhomocysteinemia (HHcy) occurs when the levels of circulating homocysteine, an intermediate in sulphur amino acid metabolism, is elevated. This elevation is linked to atherosclerosis as well as to impaired liver function (Avila et al., 2000; Zou and Banerjee, 2003; Sakuta and Suzuki, 2005; McCully, 2009). In the hepatocytes

homocysteine induced TRIB3 through the ER-stress pathway while in the endothelial cells the induction of TRIB3 expression is mediated by the cyclic adenosine monophosphate (cAMP)/response element-binding protein (CREB) pathway (Zou et al., 2011; Yu et al., 2013)

1.2.4 Inhibitors of NF-κB Mediated Transcription

Nuclear Factor-kappaB or NF-κB is the name given to a protein complex that forms dimeric transcription factors involved in the regulation of a large number of genes. This protein complex is made up of members of the Rel family of DNA-binding proteins and actively induces the transcription of genes in response to a broad range of physiological and pathological processes including the cellular inflammatory, infection and stress responses. The activation of NF-κB is connected to oncogenesis as NF-κB is known to play a role in the cell cycle and in cellular differentiation, proliferation and death (Baeuerle, 1998; Karin and Ben-Neriah, 2000; Baldwin, 2001; Ghosh and Karin, 2002; Karin and Lin, 2002). .

Both TRIB1 and TRIB3 can inhibit NF-κB mediated transcription in the cell and by doing so they modulate the immune response (Wu et al., 2003; Ostertag et al., 2010; Duggan et al., 2010). In white adipose tissue (WAT) TRIB1 expression, unlike TRIB2 or TRIB3, is elevated during both acute and chronic inflammation. This induction of TRIB1 expression in WAT under inflammatory conditions was determined to be controlled by a combination of both the NK-κB and JNK pro-inflammatory signalling axes. TRIB1 was found to inhibit the expression of a number of cytokine genes in WAT, thereby controlling adipose tissue inflammation, by direct promoter recruitment to NF-κB/RelA recognition sites in the promoter regions of these cytokines including IL-6 and TNFα (Ostertag et al., 2010).

TRIB3 expression was found to inhibit NF-κB transcription in 293T cells and in oesophageal cells TRIB3 was identified as a bile acid responsive gene that was found to control the inflammatory response by inhibition of NF-κB mediated transcription (Wu et al., 2003; Duggan et al., 2010). Further investigation in 293T cells revealed that TRIB3 can interact with the p65 subunit of NF-κB phosphorylating it and that this interaction inhibits NK-κB transcription but not the translocation of the NF-κB protein into the nucleus or its ability to bind to DNA. Due to this block in NF-κB activity in the cell, which is anti-apoptotic, TRIB3 sensitises the cell to the affects of both TNF and TNF-Related Apoptosis-Inducing Ligand (TRAIL) induced apoptosis (Wu et al., 2003).

1.2.5 Inhibitors of Retinoic Acid Receptor Signalling

Activation of the Retinoic Acid Receptor (RAR) leads to the transcription of genes containing specific DNA sequences called the retinoic acid-responsive element (RARE) in their promoter region. Genes possessing RARE DNA sequences are known to control cellular proliferation and induce cellular differentiation. All-trans retinoic acid or ATRA is a drug used in the treatment of Acute Promyelocytic Leukaemia (APL) that acts by binding to the retinoic acid-responsive elements in the DNA activating gene transcription (Kambhampati et al., 2004). Knock down of TRIB1 expression (but not TRIB3) has been shown to promote the ligand induced activation of the RARE but not the vitamin D-responsive element (VDRE) (Imajo and Nishida, 2010). It was determined that knock-down of TRIB1 significantly promoted the expression of RARβ and TRPV6 (two RAR target genes) upon ATRA treatment. TRIB1 protein was found to co-localize in the nucleus with RARα and RXRα and is able to directly interact with these two receptors in the absence or

presence of the ligand. This interaction occurs via the kinase-like domain of TRIB1 and the ligand binding domain of RARα.

1.2.6 Targets of the Cellular Stress Response

TRIB3 is up-regulated in multiple cell types in response to cellular stress including ER-stress, disrupted calcium metabolism, arsenite stress, glutathione, glutamine or glucose depletion, tropic factor deprivation, exposure to dithiothroitol and hypoxia (Mayumi-Matsuda et al., 1999; Bowers et al., 2003; Ord and Ord, 2003; Park et al., 2003; Ohoka et al., 2005; Ord and Ord, 2005; Schwarzer et al., 2006; Wennemers et al., 2011b; Qing et al., 2012; Weng et al., 2013). While ER-stress induces TRIB3 expression genotoxic stress can down-regulate TRIB3 expression (Corcoran et al., 2005). Induction of TRIB3 and other genes of the stress response pathway has been identified as a mode of action for a number of drugs or small molecules in cancer cells (Nemoto et al., 2013).

Induction of TRIB3 occurs via ATF4, which can cooperate with the C/EBP homologous protein (CHOP) to induce TRIB3 expression (Ohoka et al., 2005; Ord and Ord, 2005; Jousse et al., 2007; Rzymski et al., 2008). TRIB3 can then interact with and inhibit ATF4 and CHOP transcription activity including its own activation, and the activation of CHOP, by ATF4 (Bowers et al., 2003; Ord and Ord, 2003; Ohoka et al., 2005; Ord and Ord, 2005; Jousse et al., 2007) This raises the possibility of a negative feedback loop in the regulation of both ATF4 and CHOP activity via the induction of TRIB3.

In cancer TRIB3 expression can be induced by the stressful conditions brought about by the tumour microenvironment. TRIB3 expression in breast cancer cells (MCF7)

was found to be induced by chronic anoxic conditions via the NK-κB pathway. In these conditions the half-life of the TRIB3 mRNA was significantly increased by on average 51% when cells were transfected with the RNA binding protein Hu-antigen R (HuR) protein. TRIB3 expression was also found to be induced by ATF4 under normoxia and, to a reduced degree, and hypoxia conditions in these breast cancer cells (Rzymski et al., 2008).

1.2.7 Mediators of DNA Modification

TRIB3 expression is down-regulated by DNA damaging agents in both a p53 dependent and independent manner (Corcoran et al., 2005). TRIB3 can interact with APOBEC3A and APOBEC3C cytidine deaminases (Aynaud et al., 2012), proteins that are capable of mutating/editing viral DNA or, in the case of APOBEC3A nuclear DNA, protecting the host cell from viral infection (Navaratnam and Sarwar, 2006; Monajemi et al., 2012; Mashiba and Collins, 2013). TRIB3 is able to inhibit nuclear DNA editing by APOBEC3A protecting the cell against APOBEC3A induced double-stranded DNA breaks (Aynaud et al., 2012)

1.2.8 Targets of Wnt/TCF Signalling

The Wnt signalling pathway begins with the binding of one of the 19 members of the Wnt protein family to the extracellular domain of a Frizzled (Fz) family receptor. This leads to the activation of the Wnt signalling pathway within the cell, a pathway whose activation plays a crucial role regulating diverse cellular processes such as cellular proliferation, migration, differentiation and survival. Canonical Wnt signalling is β-catenin (CTNNB1)-dependent. Activation of the Wnt signalling pathway leads to the accumulation of CTNNB1 in the cell; CTNNB1 then enters the

nucleus where it acts a transcriptional switch, co-activating TCF/LEF-mediated transcription. Wnt pathway mutations are frequently observed in cancer often resulting in hyperactivation of the Wnt signalling pathway. Aberrant Wnt pathway signalling has been linked to a large number of cancers including liver, ovary and pancreatic cancer as well as to leukaemia (Memarian et al., 2012; Anastas and Moon, 2013).

TRIB2 expression was linked to the Wnt pathway when Wnt1 expression in a hepatoma cell line (Huh7) was found to increase β-catenin nuclear accumulation and TRIB2 expression (J. Wang et al., 2013). TRIB2 expression was found to be regulated by both FoxA1 and TCF4 in these liver cancer cells. The FoxA factors regulate Wnt/TCF-mediated transcription in liver cancer cells and FoxA1 and TCF4 were found to co-occupy a TRIB2 distal enhancer region in the TRIB2 intron. While FoxA1/2 binding to the TRIB2 promoter is independent of Wnt/TCF it is critical for TCF4 binding to this region (J. Wang et al., 2013).

Wnt signalling was also determined to regulate the levels of Yes-Associated Protein (YAP), an intracellular transducer of signalling of the tumour suppressor Hippo, in liver cancer cells. Increased YAP expression has been shown to cause liver cancer in mice (Dong et al., 2007; Lu et al., 2010). TRIB2 depletion in HepG2 cells was determined to inhibit the transcription of Connective Tissue Growth Factor (CTGF) and Ankyrin Repeat Domain-Containing Protein 1 (ANKRD1), both YAP target genes. TRIB2 was found to regulate YAP via regulation of YAP protein stabilization. TRIB2 increased YAP protein stabilization through direct interaction with the βTrCP ubiquitin complex (a subunit of the S-Phase Kinase-Associated Protein 1 (SKP1)- Cullin(CUL)1-F-Boc E3 ubiquitin ligase involved in YAP proteasomal degradation) via its C-terminus (J. Wang et al., 2013).

TRIB2 was also found to regulate YAP protein activity via depletion of C/EBPα in the liver cancer cells. C/EBPα expression was found to inhibit a YAP dependent Transcriptional Enhancer Factor TEF (TEAD) reporter and to down-regulate CTGF. C/EBPα directly bound to the YAP protein and was found to inhibit the ability of YAP to bind TEAD4 thereby regulating YAP-mediated downstream transcription activity (J. Wang et al., 2013). TRIB2 expression can therefore increase YAP activity in the liver cancer cell by way of depletion of C/EBPα protein.

1.3 The Tribble and Haematopoiesis

1.3.1 Haematopoiesis

Haematopoiesis is the formation of the blood, the liquid tissue of the body. An average adult human body contains between five and six litre of blood which is made up of both liquid (plasma) and cellular portions. Blood is responsible for a vast array of function important for maintaining life including the mediation of metabolic interactions among all tissues in the body. It is also responsible for the transport of nutrients and waste products around the body. Another major function of the blood is the transport of oxygen (O_2) and carbon dioxide (CO_2), as well as hormonal signals, through the body.

About half of the blood volume is comprised of blood cells and cellular fragments. The cellular component of the blood can be split into three major types. First there are the erythrocytes (red blood cells), these cells lack a nucleus and are specialised in the transport of O_2 and CO_2. The second cell type that make up the blood are the leukocytes (white blood cells), these cells are integral to the body's immune system and are outnumbered by the erythrocytes be about 1,000 to one. Finally there are the

platelets which are cellular fractions responsible for blood clotting. The lifespan of all these cells differ and can range from about 120 days for erythrocytes to years for some types of leukocytes (e.g. memory T cells) (Lehninger et al., 2004; Lensch, 2012).

Unlike erythrocytes or platelets there are many distinct types of leukocytes. Traditionally the leukocytes are divided into two distinct lineages. Firstly there is the myeloid lineage which consists of a large number of functionally and morphologically diverse cell types. The myeloid lineages include the granulocytes (neutophils), the monocytes, the eosinophils, the basophils and the megakaryocytes (which produce platelets) as well as the erythrocytes. According to the classic model of haematopoiesis all cells of the myeloid lineage are derived from a common progenitor cells called the common myeloid progenitor (CMP) (figure 1.2). The myeloid cells are integral to the immune response being involved, for example, in the mediation of the inflammatory response and in the phagocytosis of invading microorganisms (Alberts et al., 2002; Abul K. Abbas et al., 2007; Iwasaki and Akashi, 2007; Weissman and Shizuru, 2008).

The second lineage of haematopoiesis is the lymphoid lineage. Like the myeloid lineage these cells are classically thought to be derived from a common progenitor cell, the Common Lymphoid Progenitor (CLP) (figure 1.2). There are three types of lymphocytes; T, B and natural killer (NK) cells which are also vital to the immune response as they are responsible for the production of antibodies and the killing of tumour and virus infected cells (Alberts et al., 2002; Abul K. Abbas et al., 2007; Iwasaki and Akashi, 2007; Weissman and Shizuru, 2008).

The dendritic cells are antigen-presenting cells that act as a bridge between the innate and adaptive immune response. They are unique in that it is thought that they can develop from either the myeloid or the lymphoid pathways (figure 1.2) (Alberts et al., 2002; Abul K. Abbas et al., 2007; Iwasaki and Akashi, 2007; Weissman and Shizuru, 2008).

All cells of the blood are maintained by the haematopoietic stem cell (HSC) which is found in the bone marrow (figure 1.2). Discovered in 1961 (Till and McCulloch, 1961, 2012) the HSCs can undergo self-renewal and are capable of differentiating into all the different cells of the haematopoietic system. The development of all blood cells, apart from the T cells, occurs in the bone marrow. Uniquely the T cells mature in the thymus from progenitor cells that arise in the bone marrow. In the classical model of haematopoiesis the HSC were thought to differentiate first into the common myeloid or lymphoid progenitor cells, splitting the leukocytes into the myeloid and lymphoid populations (Abul K. Abbas et al., 2007; Iwasaki and Akashi, 2007). Current knowledge now suggests that the HSC cells can give rise to multipotent progenitor (MPP) cells that possess a differentiation bias towards either myeloid or lymphoid fates (figure 1.2). MPP cells with a differentiation bias towards the myeloid fate generally differentiate into CMP cells, though this MPP can still differentiate into lymphoid progenitor cells. The CMP cell can further differentiate into the megakaryocyte-erythrocyte progenitor (MEP) or granulocyte-monocyte progenitor (GMP) cells (figure 1.2). MEP cells, which can also arise directly from the MPP cells, go on to form the erythrocytes and megakaryocytic cells while the GMP progenitor cells gives rise to the granulocytes (neutophils), the monocytes, the eosinophils, the basophils as well as potentially the dentritic cells (figure 1.2) (Iwasaki and Akashi, 2007; Weissman and Shizuru, 2008; Lensch, 2012).

MPP cells with a differentiation bias towards the lymphoid state gives rise to the lymphoid-primed multipotent progenitor (LMPP) cells (while still retaining the potential to form CMP cells) (figure 1.2). These LMPP cells can further differentiate into with the GMP cells or into the CLPs which give rise to the B-, T- and Natural Killer (NK) cells as well as, potentially, the dentritic cells (figure 1.2) (Iwasaki and Akashi, 2007; Weissman and Shizuru, 2008; Lensch, 2012).

All these various progenitor cells will undergo differentiation until, finally, the cells undergo terminal differentiation into the various different mature cells of the blood (figure 1.2) (Iwasaki and Akashi, 2007; Weissman and Shizuru, 2008; Lo Celso et al., 2009; Kondo, 2010; Lensch, 2012). Cytokines promote the proliferation and maturation of the HSC and are necessary for normal haematopoiesis. Cytokines are thought to play two possible roles in haematopoiesis. By providing lineage specific signals to the progenitor cells they may play an instructive role. However they could also play a supportive (stochastic) role by stimulating proliferation and supporting the survival of the progenitor cells. Cytokines are produced by the cells of the bone marrow thereby providing an environment for haematopoiesis. Cytokines generated during the immune response also stimulate haematopoiesis providing a mechanism by which the bone marrow is able to tailor leukocytes production in response to infection (Abul K. Abbas et al., 2007; Kondo, 2010). The control of transcription factor expression, either by extra- or intra-cellular signalling, is crucial to maintaining normal haematopoiesis. Expression of individual transcription factors such as Pu.1, GATA-1 and C/EBPα are known to drive the formation of specific cell types and the dysregulation of the expression of these transcription factors perturbs haematopoiesis (Iwasaki and Akashi, 2007).

The above information was largely derived from mouse model, which is vital for the investigation and understanding of haematopoiesis. However mice and humans differ in size, ecology, lifespan, and age to reproductive maturity. Human populations are also more genetically diverse compared to the inbred mouse models. All this can affect the proliferative demands on stem and progenitor cells making investigation into human haematopoiesis vital to understanding leukaemiogenes.

All the different cellular populations of haematopoiesis are defined by the presence of specific cell surface markers. In order to purify different cellular populations the simultaneous detection of several independent cell surface markers is used. Comparison of surface markers indicates that similar haematopoietic cells populations generally lack common surface marker types between humans and mice. For example human HSCs express the FLT3 receptor while murine HSCs cells do not (Sitnicka et al., 2003; Doulatov et al., 2012). Based on cell surface markers the haematopoietic lineage of humans has been investigated. Unlike mice, whose HSC have been separated into long-term (LT), intermediate-term (IT), and short-term (ST) cell populations based on cell surface markers, heterogeneity of HSC cells have not been investigated (figures 1.2 and 1.3). In humans only one population of immature lymphoid progenitors (MLPs) has been identified (figure 1.3). These MLP cells retain the ability to mature into monocytes as well as lymphoid cells (figure 1.3). The CMP, MEP and GMP populations have been identified in both mice and humans (figure 1.3). Genes such as Notch1, C/EBPα, PU.1 and GATA-2 have been identified as important regulators of lineage commitment in human haematopoiesis (Doulatov et al., 2012).

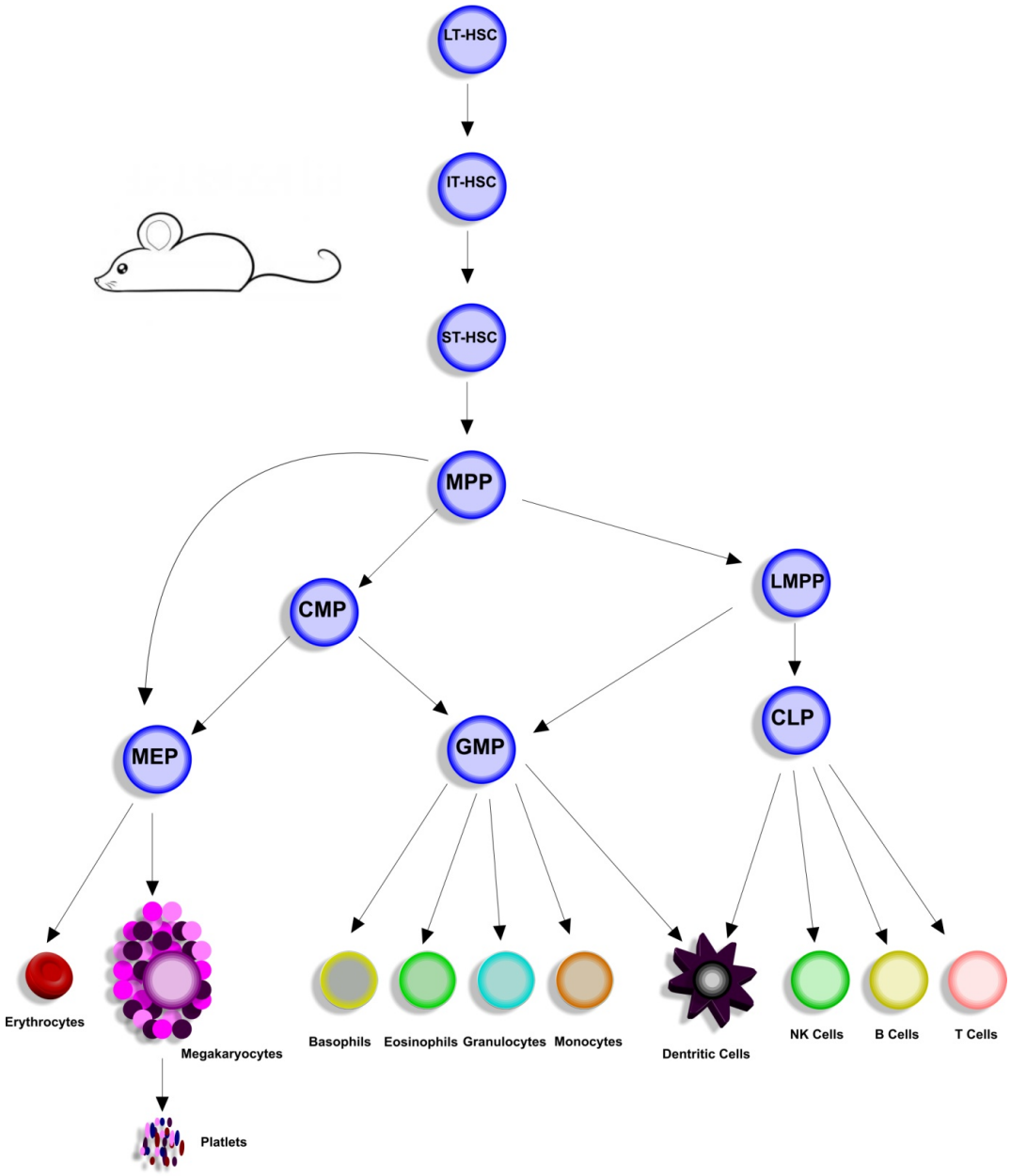

Figure 1.2: Current model of haematopoiesis in mice Diagram of haematopoietic development from the haematopoietic stem cells (HSCs) at the top of the diagram through the major classes of progenitor cells described in the text to the terminally differentiated cells at the bottom. The inferred differentiation pathways are depicted by the arrows ((Iwasaki and Akashi, 2007; Weissman and Shizuru, 2008; Doulatov et al., 2012)). LT-HSC = Long Term HSC; IT-HSC = Intermediate-term HSC; ST-HSC = Short-term HSC; MPP = Multipotent progenitor; CMP = Common Myeloid Progenitor; GMP = Granulocyte-Monocyte Progenitor; MEP = Megakaryocyte-Erythrocyte Progenitor; LMPP = Lymphoid-Primed Multipotent Progenitor; CLP = Common Lymphoid Progenitor; NK Cells = Natural Killer Cells.

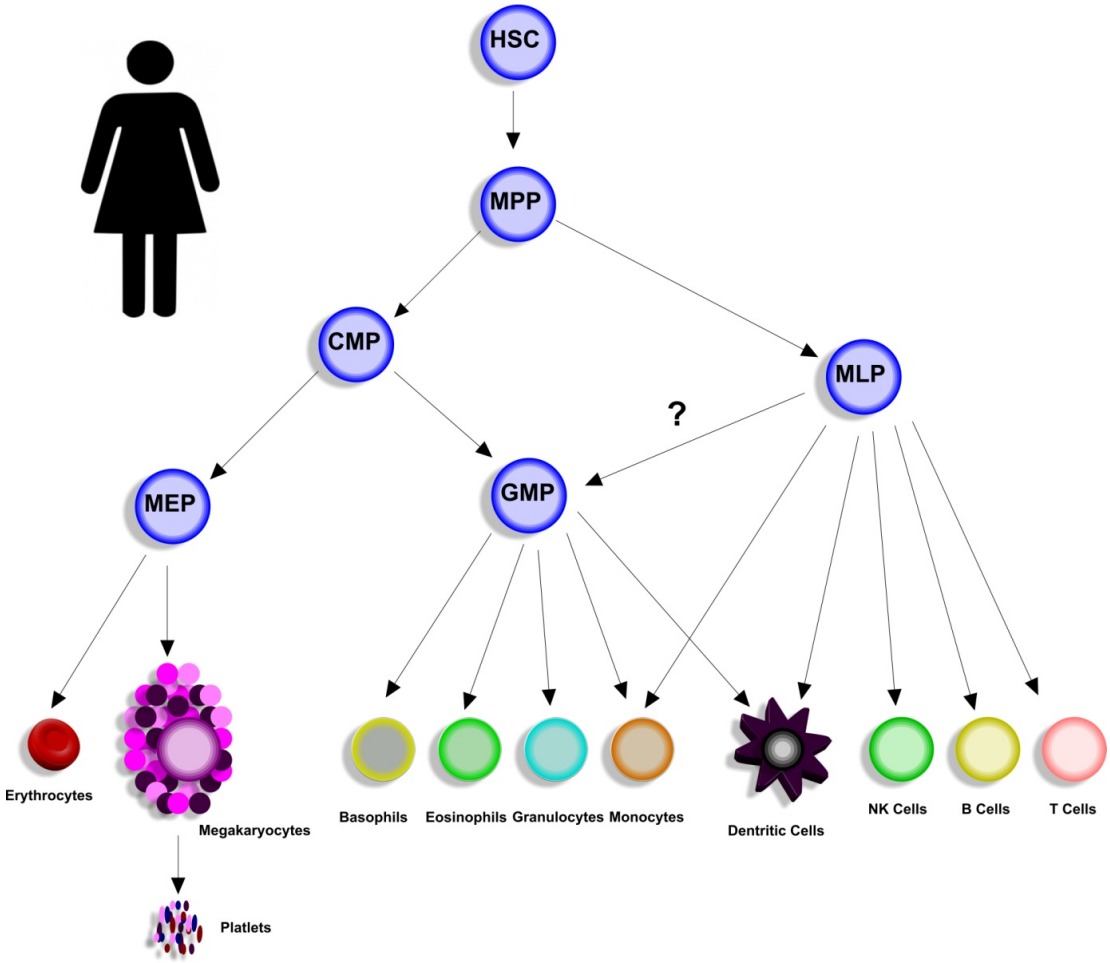

Figure 1.3: Current model of haematopoiesis in humans. Diagram of haematopoietic development from the haematopoietic stem cells (HSCs) at the top of the diagram through the major classes of progenitor cells described in the text to the terminally differentiated cells at the bottom. The inferred differentiation pathways are depicted by the arrows. The question mark indicates an inferred but yet to be experimentally determined pathway in human haematopoiesis based on knowledge from murine haematopoiesis. Information for diagrams derived from (Iwasaki and Akashi, 2007; Weissman and Shizuru, 2008; Doulatov et al., 2012). HSC = Haematopoietic Stem Cells; MPP = Multipotent progenitor; CMP = Common Myeloid Progenitor; GMP = Granulocyte-Monocyte Progenitor; MEP = Megakaryocyte-Erythrocyte Progenitor; MLP = Immature Lymphoid Progenitor; NK Cells = Natural Killer Cells.

1.3.2 Tribble in Normal Haematopoiesis

While over-expression of Trib1 and Trib2 induces leukaemia in mice (Keeshan et al., 2006; Jin et al., 2007; Dedhia et al., 2010) the role the Tribble genes play in normal haematopoiesis is poorly understood.

Analyses of Trib1-deficient mice does show that they lack tissue-resident M2-like macrophages (associated with responses to anti-inflammatory reactions and tumour progression) and eosinophils due to aberrant C/EBPα expression (Satoh et al., 2013) suggesting that Trib1 expression is a key factor in the development of these myeloid cells. Analyses of Trib2 and Trib3 deficient mice however revealed no defects in either the myeloid or lymphoid cells (Satoh et al., 2013). This lack in defects may be explained by the overlapping function of the Tribble genes, for example both Trib1 and Trib2 can degrade C/EBPα (Dedhia et al., 2010) and Trib2 and Trib3 can interact with and inhibit AKT phosphorylation (Du et al., 2003; Ding et al., 2008; Xie et al., 2012) suggesting that the genes may have redundant functions.

Mice lacking Trib2 expression show no defects in heamatopoesis. However aberrant Trib2 expression in mice was found to perturb myeloid cell differentation. Increased Trib2 expression lead to a decrease in the granulocytic population and an increase in the monocytic population as well as an increase in the numbers of cluster of differentiation(CD)11b+CD11c+MHCII+ dendritic cells and CD11b+F4/80+MHCII+ macrophages in these mice (Keeshan et al., 2006). Further analysis revealed that expression of either Trib1 or Trib2 (but not Trib3) is able to resist granulocyte colony-stimulating factor (G-CSF) induced granulocytic differentiation of 32D cells, a myeloid cell line. The ability of Trib1 and 2, but not

Trib3, to degrade C/EBPα in the cell is thought to be the reason for this perturbation in myeloid differentiation (Dedhia et al., 2010; Keeshan et al., 2010).

In human heamatopoesis the Tribble genes were found to be differentailly expressed in the different cellular compartments of the heamatopoetic system (K. L. Liang et al., 2013). TRIB1 expression was found to be significantly increased in the Granulocyte/Monocyte compartment and the B cell compartment compared to other lineages. TRIB2 expression was significantly increased in the T cell compartment (K. L. Liang et al., 2013) (data also published by us in the British Journal of Haematology 2012, see Appendix A) and significantly higher in the CD4+ (helper T cells) versus the CD8+ (Cytotoxic T cells) T cells. TRIB3 expression was found to be significantly higher in the erythrocyte compartment (K. L. Liang et al., 2013).

1.3.3 Tribble and the Immune Response

Each of the Tribble genes have been reported to be expressed in various cells of the immune system (the leukocytes) suggesting that Tribble expression may play a key role in the function of the leukocytes and the immune response. TRIB1 is expressed in monocytes, in macrophage cells, in a murine leukemic monocyte macrophage cell line (RAW254.7) and in antigen-presenting and activated endothelial cells (Ashton-Chess et al., 2008; Eder et al., 2008a; Deng et al., 2009; Y.-H. Liu et al., 2013). TRIB2 has been found to be expressed in monocytes (Eder et al., 2008b). Finally TRIB3 is expressed in the RAW254.7 cell line, in monocytes and in mast and microglial cells (Eder et al., 2008a; Nagarkatti et al., 2009; Ord et al., 2012; Y.-H. Liu et al., 2013). Analyses of Tribble expression and function in these cells link the Tribble genes to the regulation and modulation of the immune response

Stimulation of monocytes with lipopolysaccharides (LPS) affects Tribble expression and Low-Density Lipoprotein (LDL) uptake in these cells. TRIB1 and TRIB3 expression has been reported to be up-regulated at three and nine hours respectively (Eder et al., 2008a) post LPS stimulation while TRIB2 expression in monocytes was reduced by LPS stimulation (Eder et al., 2008b). Depletion and over-expression of each of the Tribble proteins had no affect on LPS mediated acetylated low-density lipoprotein or acLDL uptake by the monocytes suggesting that the Tribble proteins do not mediate LDL uptake itself (Eder et al., 2008a). TRIB2 was found to interact with members of the Mitogen-Activated Protein Kinase (MAPK) signalling pathway leading to the suppression of Interleukin (IL) -8 production. Depletion of TRIB2 expression led to an increase in IL-8, a cytokine produced by monocytes in response to LPS suggesting that TRIB2 is a novel regulator of the inflammatory activation in response to inflammatory signals (Eder et al., 2008b).

Trib1 expression was found to control the migration of mouse monocyte cells. Trib1 is expressed in RAW264.7 cells where depletion of Trib1 expression was found to affect both the morphology and migration potential of the cells causing them to exhibited a more monocyte-like morphology. Trib1 expression was found to be induced by Interferon Gamma (IFN-γ) and Toll-like receptor (TLR) 2 ligands, both modulators of the immune system, in the cell and Trib1 expression positively regulated Extracellular Signal-Regulated Kinase (ERK) 1 and ERK2 phosphorylation in response to treatment with IFN-γ and TLR2 ligands. Depletion of Trib1 expression in this macrophage cell line resulted in an increase in the expression of C/EBPβ and Tumor Necrosis Factor (TNF) -α (which is secreted by activated RAW cells in response to IFN-γ stimulation (Vila-del Sol et al., 2008))

associating Trib1 expression with the attenuation of TNF-α production. (Y.-H. Liu et al., 2013).

TRIB3 expression has been linked to the activity and survival of mast cells linking TRIB3 expression to the modulation of the immune system (Kuo et al., 2012; Ord et al., 2012). The expression of TRIB3 was found to be positively regulated by IL-3 in bone marrow-derived mast cells (BMMCs). While $TRIB3^{-/-}$ BMMCs did not show any difference in IL-3 induced maturation compared to their wild-type counterparts they were more sensitive to IL-3 deprivation showing increased apoptosis and inhibition of growth. The activation of $TRIB3^{-/-}$ BMMCs was also found to be impaired; the BMMCs showed impaired degranulation and induction by cytokines (Ord et al., 2012). TRIB3 expression in sensitized mast cells inhibits the production of a number of cytokines and chemokines including IL-4 and IL-6 indicating that TRIB3 plays a role in the down-regulation of the inflammatory response (Kuo et al., 2012)

Cannabinoids have been reported to exert immunosuppressive and anti-inflammatory affects on the body (Nagarkatti et al., 2009). Cannabidiol up-regulates TRIB3 and Activating Transcription Factor 4 (ATF4) target genes in LPS-activated BV-2 microglial cells; inducing the cellular stress response. This suggests that increased TRIB3 expression may be linked to immunosuppressant activity in the body (Juknat et al., 2013). TRIB3 was also found to be a target of the Fibrates, a class of a class of amphipathic carboxylic acids used to treat hyperlipidemia and to prevent the progression of atherosclerotic lesions (Chapman, 2003). Recent evidence suggests that Fibrates prevent the progression of atherosclerosis by exerting immunomodulatory affects on atherosclerotic plaques (Duez et al., 2001; Cunard, 2005). Fibrates, which are ligands of the Peroxisome Proliferator-Activated Receptor

(PPAR) α receptor, were found to induce TRIB3 expression in lymphocytes independent of PPARα. Induction of TRIB3 expression in the lymphocytes lead to cell cycle G2 arrest and increased apoptosis via reduction of the expression of Cyclin B1 (Selim et al., 2007; Morse et al., 2009), suggesting that TRIB3 expression is immunomodulatory.

TRIB1 expression is altered upon Regulatory T (Treg) cell activation suggesting that TRIB1 may play a role in the Treg response. T regs are a subpopulation of T cells involved in the suppression of auto-reactive T cells, B cells, natural killer cells, natural killer T cells, mast cells, and dendritic cells. As they suppress these cells they are involved in peripheral self-tolerance and depletion of T regs leads to autoimmunity (Gorantla et al., 2010). TRIB1 expression is transiently increased for 1 to 2 hours after T reg activation. TRIB1 expression was also observed to be higher in both human and murine CD4+CD25+CD127-Tregs cells compared to their CD4+CD25-non-Tregs cell counterparts. Increased TRIB1 expression blocked T cell proliferation suggesting that TRIB1 plays a key role in Treg function (Emilie Dugast et al., 2013).

1.4 Other Functional Roles of the Tribble

1.4.1 Tribble in Development

Various knock-down and misexpression studies of the drosophila Tribble or trbl gene showed that trbl has highly varied tissue specific functions in development. Trbl coordinates cell proliferation and migration in the developing drosophila and Xenopus embryos and has also been shown to control cell division during tissue pattering (Dobens and Bouyain, 2012).

The trbl protein was found to block mesoderm proliferation by direct turnover of String phosphatise (a CDC25 homolog). A double mutation of both Sting and Trbl lead to normal gastrulation. Snail was also identified as a co-activator of Trbl in Drosophila necessary for its ability to block mesoderm proliferation (Grosshans and Wieschaus, 2000). As previously discussed, over-expression of Trbl results in the degradation of the Drosophila C/EBP homologue slbo; this leads to a block in migration of border cells during Drosophila development. Slbo can also suppress trbl expression in the cell and the kinase domain of trbl is necessary for the interaction between them (Rørth et al., 2000; Masoner et al., 2013). Over-expression of Trbl also leads to premature mitosis in the mesoderm cells by specific degradation of String and Twine via the proteosome, both of which are Cell Division Cycle 25 (CDC25) mitotic activators (Mata et al., 2000; Farrell and O'Farrell, 2013).

1.4.2 Tribble as Mediators of Apoptosis

Members of the Tribble family are involved in the regulation of cell survival and death. Expression of the Tribble family members have been found to both induce and protect against apoptosis. Whether their expression promotes or protects against cell death has been found to be both cell and context specific.

1.4.2.1 Pro-apoptotic Function of the Tribble

A number of publications have linked TRIB2 expression to the induction of apoptosis. In TF-1 cells, an erythroleukemic cell line, survival factor withdrawal was found to induce apoptosis via a TRIB2-Mcl-1 dependant pathway in TF-1 cells (Lin et al., 2007) and in Me-1 leukemic cells both the expression of TRIB1 and TRIB2 was capable of inhibiting JNK *(*c-Jun N-terminal Kinase) activation and was found

to be both pro-apoptotic and growth restrictive (Gilby et al., 2010). In fact the *e*ctopic expression of TRIB2 was found to induced apoptosis in many leukemic cell lines although U937 and K562 proved to be resistant to apoptosis induced by TRIB2 expression along with a number of non-haematopoietic cell lines (Lin et al., 2007).

TF-1 cells are dependent on human granulocyte macrophage colony-stimulating factor (GM-CSF). TRIB2 was identified as an immediate early gene that was induced in TF-1 cells by GM-CSF deprivation and depletion of TRIB2 led to a reduction of apoptosis in TF-1 cells post GM-CSF withdrawal, a response also observed in CD4+ T cells. This induction of TRIB2 expression was found to be highly selective; other apoptotic signals such as ultraviolet (UV) irradiation did not induce TRIB2 expression (Lin et al., 2007).

TRIB2 was found to trigger apoptosis via mitochondria dysfunction in these TF-1 cells; TRIB2 activated the caspase proteins caspase-3, caspase-8 and caspase-9 expression, to activate Bcl-2-Associated X (BAX), a pro-apoptotic molecule, and to induce the release of cytochrome C from the mitochondria into the cytoplasm. TRIB2 expressions lead to the cleavage of Mcl-1, a pro survival protein, via a caspase-dependent but proteosomal independent pathway. Over expression of Mcl-1 (or Bcl-2) was able to inhibit TRIB2 induced apoptosis in the cell (Lin et al., 2007).

TRIB3 has also been identified as a mediator of apoptosis in the cell acting as a link between ER-stress induced C/EBP Homologous Protein (CHOP) expression and subsequent apoptosis of the cell (Ohoka et al., 2005). TRIB3 is cleaved by multiple caspase proteins both *in vitro* and during cellular apoptosis, this cleavage was found to promote apoptosis. (Tadokoro et al., 2010; Shimizu et al., 2012). TRIB3 expression has also been shown to sensitise breast carcinoma cells to hypoxia

induced death by repression of the NF-κB pathway and ATF4 transcription in the cell (Rzymski et al., 2008).

1.4.2.2 Anti-apoptotic Function of the Tribble

Though both TRIB2 and TRIB3 have been shown to act in a pro-apoptotic manner in the cell, both have also been shown to act in an anti-apoptotic manner. For example over-expression of TRIB2 in lung cancer cells has been associated with a reduction of apoptosis (Grandinetti et al., 2011). Over-expression of TRIB3 can also play an anti-apoptotic roll in the cell as it can protect prostrate carcinoma cells from starvation induced apoptosis. (Schwarzer et al., 2006). This indicates that TRIB3 may assist the growth of tumour cells under stressful conditions and in fact under ER-stress conditions the TRIB3 protein becomes resistant to caspase induced cleavage. Full length TRIB3 protein is also able to repress caspase-3 (CASP3) activity by forcing the relocation of the proCASP3 protein to the nucleus inhibiting its ability to induce apoptosis (Shimizu et al., 2012).

Altogether this information shows that both TRIB2 and TRIB3 can act in both a pro- and anti-apoptotic manner in the cell. Whether these genes act one way or the other may be cell type or even context specific as evidenced by the TRIB3 protein which can resist caspase cleavage under ER-stress conditions (Shimizu et al., 2012).

1.5 Tribble and Disease

1.5.1 Tribble and Solid Cancer

Investigation into the solid tumours has indicated that over-expression of the Tribble proteins may contribute to the survival, growth and invasion potential of solid tumours.

1.5.1.1 TRIB1 in Solid Tumours

Over-expression of TRIB1 has been observed in epithelial ovarian cancer, breast cancer, follicular thyroid cancer and rectal cancer (Puskas et al., 2005; Puiffe et al., 2007; Bhushan and Kandpal, 2011; J.-W. Liang et al., 2013). Increased TRIB1 expression in the ascites of patients with ovarian cancer was found to be associated with poor survival. When epithelial ovarian cancer cells (OV-90 cells) were cultured with the acellular fraction of stimulatory ovarian cancer-derived ascites that increase invasion potential of OV-90 cells this lead to the induction of TRIB1 expression (Puiffe et al., 2007).

Ephrin Type-B Receptor 6 (EphB6) is a receptor tyrosine kinase whose expression has been implicated in several cancers. Expression of EphB6 is often found to be silenced in invasive breast carcinoma. TRIB1 and TRIB3 expression is down-regulated in breast carcinoma cells transfected with EphB6. (Bhushan and Kandpal, 2011).

In rectal cancer gains in the 8q24.3 TRIB1 containing locus and increased TRIB1 expression associated with this gain has been observed (J.-W. Liang et al., 2013). Chromosomal amplification of 8q24 in oesophageal cancer was not associated with

increased TRIB1 expression indicating that TRIB1 is not the target of this amplification (Huang et al., 2006). Indicating that oncogenic activity of TRIB1 may depend on cell type.

1.5.1.2 TRIB2 in Solid Tumours

Elevated levels of TRIB2 have been associated with melanoma, lung cancer, liver cancer, osteosacoma and prostate cancer (Zanella et al., 2010; Grandinetti et al., 2011; Schoolmeesters et al., 2012; Zhang et al., 2012; P.-Y. Wang et al., 2013). Elevated levels of TRIB2 expression were found in human skin cancer patient samples as well as in the melanoma cell line G-361. TRIB2 was identified as a repressor of Forkhead Box Protein O (FOXO), a transcription factor and tumour suppressor which contributes to the malignant phenotype of melanoma cells. TRIB2 expression was also determined to facilitated the growth and survival of melanoma cells (Zanella et al., 2010). TRIB2 was found to be over-expressed in ~30% of human lung cancer tumour samples, with 12% of samples possessing gene amplification surrounding the TRIB2 locus (Grandinetti et al., 2011). Of interest is the fact that a number of lung tumour samples which were found to have low TRIB2 expression also contained a subset of cells that exhibited high TRIB2 expression; the authors of the study theorised that this subset of cells with TRIB2 over-expression may represent the tumour-initiating cells of these tumours. In addition analysis of non-small cell lung cancer (NSCLC) cell lines analysed showed over expression of TRIB2 when grown under spheroid conditions. (Grandinetti et al., 2011).

Compelling evidence indicates that TRIB2 dysregulation also plays a role in lung cancer proliferation and survival. Depletion of TRIB2 expression in NSCLC cell lines, which also correlates with an increase in C/EBPα expression, increases

apoptosis and decrease cell proliferation. This affect was not observed in normal lung fibroblast and epithelial cells (Grandinetti et al., 2011). Let-7c, an mRNA that is down-regulated in lung cancer tissues and the micro-RNAs miR-551 and miR-1297 all suppress lung cancer cell proliferation, induce apoptosis and hinder xenograft tumour growth by inhibiting TRIB2 expression. Let-7c, miR-551 and miR-1297 expression was linked to an increase in C/EBPα. Expression and Let-7c also increases p-p38MAPK in lung cancer cells (Zhang et al., 2012; P.-Y. Wang et al., 2013). Indeed, mice injected with the NSCLC cell lines with depleted TRIB2 expression remain tumour free, unlike those injected with mock transfected NSCLC cells. When knock-down resistant TRIB2 was used to rescue the TRIB2 expression in the NSCLC cell lines with depleted TRIB2 expression these cells caused rapid tumourigenesis upon injection into mice (Grandinetti et al., 2011).

TRIB2 expression was determined to be critical for liver cancer cell survival and proliferation. TRIB2 was identified as a Wnt-specific target gene acting downstream of the Wnt/TCF signalling pathway in liver cancer. TRIB2 inhibition leads to decreased cell proliferation, impaired colony formation ability and increased apoptosis in HepG2 liver cancer cells. (J. Wang et al., 2013). TRIB2 is also the target of other tumour promoting genes in the cell. TRIB2 mRNA was found to be regulated by TNFα, a tumour promoter in osteosacoma and prostate cancer cell lines.

1.5.1.3 TRIB3 in Solid Tumours

To date TRIB3 expression has been associated with glioma, breast, lung, liver, oral tongue squamous cell carcinoma and colon cancer (Miyoshi et al., 2009; Salazar et

al., 2009b; Lorente et al., 2009; J. Zhang et al., 2011; Bhushan and Kandpal, 2011; Wennemers et al., 2011a, 2011b, 2012; Salazar et al., 2013; Vara et al., 2013; Zhou et al., 2013; Izrailit et al., 2013; Li et al., 2013). The role played by TRIB3 in the development and maintenance of cancer is less clear than that of TRIB1 or TRIB2. Forced over-expression of Trib3 in murine bone marrow does not lead to the development of AML (Dedhia et al., 2010) and while TRIB3 expression has been found to promote tumour cell survival and associated with poor prognosis (Miyoshi et al., 2009; Wennemers et al., 2011b; Izrailit et al., 2013; Li et al., 2013) it has also been associated with good prognosis and the induction of apoptosis in the tumour cell (Wennemers et al., 2011a; Salazar et al., 2013; Vara et al., 2013). In human non-small cell lung cancer increased TRIB3 expression correlates with tumour metastasis, disease recurrence and poor survival in patients (Zhou et al., 2013). Depletion of TRIB3 in lung cancer cells leading to a reduction in Notch1 expression and the inhibition of cellular proliferation and invasion, a reduction in tumour metastasis and growth and an increase in apoptosis (Zhou et al., 2013).

Induction of autophagy by TRIB3 was found to play a pro-survival role and attenuated the apoptotic cascade in human non-small cell lung cancer cells. Salinomycin, a novel agent that can selectively eradicate breast and other cancer stem cells (Gupta et al., 2009), has been shown to induce autophagy in human non-small cell lung cancer cells via an ATF4-DNA Damage-Inducible Transcript 3(DDIT3)/CHOP-TRIB3-AKT1-MTOR axis (Li et al., 2013). Salinomycin induced the expression of TRIB3 via the ATF4-DDIT3 pathway in the cell, TRIB3 was then able to repress AKT1 and subsequently Mammalian Target of Rapamycin (MTOR) (a central regulator of autophagy induction (Maiuri et al., 2007; Mehrpour et al., 2010)). While induction of autophagy plays a pro-survival role in lung cancer cells,

in other cancer cells induction of TRIB3 mediated autophagy leads to increased rates of apoptosis. TRIB3 is the link between the ER-stress response and anti-tumoural autophagy induced by cannabinoids in cancer cells including both hepatocarcinoma and glioma cells (Salazar et al., 2009b, 2009a, 2013). In hepatocarcinoma cells TRIB3 expression necessary for PPARγ activation and subsequent cannabinoid-induced autophagy (Vara et al., 2013). The cannabinoid Δ^9-tetrahydrocannabinol or THC was shown to inhibit AKT and subsequent mTOR activation via TRIB3 (Salazar et al., 2013). Further investigation has shown that transformed TRIB3 deficient cells are resistant to cannabinoid-induced cell death mediated by autophagy and, that TRIB3 deficient tumours are resistant to the anticancer action of cannabinoid (Salazar et al., 2013). In glioma cells increased amphirregulin expression protects the cells from cannabinoid induced autophagy by inhibiting the expression of TRIB3 (Lorente et al., 2009).

Other drugs induce liver cancer cell death via the induction of TRIB3 expression. The combination of dehydroxymethyl-epoxyquinomicin (DHMEQ) and Celecoxib or of MG132 and Celecoxib cooperate together to kill hepatocarcinoma cells via the induction of stress response proteins such as TRIB3 (Lampiasi et al., 2009; Cusimano et al., 2010; Lampiasi et al., 2012). Both Oroxylin A and Wogonin, an anticancer flavonoid, are also able to induce cytotoxicity in human hepatocellular carcinoma cells via induction of TRIB3 and other genes involved in unfolded protein response leading to the inhibition of AKT activation (Xu et al., 2013, 2012).

As previously stated both TRIB1 and TRIB3 expression was found to down-regulated in breast carcinoma cells transfected with EphB6 (Bhushan and Kandpal, 2011). TRIB3 has also been identified as a positive regulator of the Notch ligand Jagged 1 (JAG1), a marker for relapse and poor outcome in breast cancer (Reedijk et

al., 2005), via both the MAPK-ERK and TGFβ/SMAD4 signalling pathways in human mammary carcinoma cell (MDA MB231 cells). TRIB3 deficiency in the cells leads to a decrease in proliferation, which was rescued by JAG1 expression, and a decrease in growth of mouse xenografts of the MDA MB231 cells (Izrailit et al., 2013). TRIB3 is also one of the ER-stress response genes induced gamma-tocotrienol, a vitamin E compound that induces apoptosis in mammary tumour cells (Wali et al., 2009).

However the role TRIB3 plays in breast cancer is not clear. TRIB3 mRNA expression has been associated with poor prognosis in this cancer, But TRIB3 protein expression has been associated with good prognosis with little correlation between TRIB3 protein and mRNA expression. Therefore TRIB3 expression in breast cancer is associated with both negative and positive outcomes depending on whether it is protein or mRNA expression that is measured (Wennemers et al., 2011a, 2011b). Investigation into the lack of correlation between TRIB3 mRNA and protein levels revealed firstly that the TRIB3 protein, which is not degraded by the proteosome or through autophagy, is very stable and has a half life comparable to α-tubulin in both normoxia and hypoxia conditions. Translation efficiency of the TRIB3 mRNA into protein in breast cancer cells was also found to be affected in anoxic conditions. Anoxia was found to inhibit TRIB3 translation from mRNA into protein, though TRIB3 mRNA levels are increased by hypoxic stress (Wennemers et al., 2011b, 2012).

Apart from anoxia a variedly of different stresses increase TRIB3 expression in breast cancer cell lines, these include endoplasmic reticulum (ER) stress and nutrient starvation. TRIB3 mRNA is also induced by hypoxia in breast cancer cells, xenografts and breast cancer tissue. However TRIB3 expression itself was

determined not to be a hypoxia marker in breast cancer samples (Wennemers et al., 2011b). The induction of TRIB3 mRNA in response to stress in breast cancer cell lines occurred via PRKR-Like Endoplasmic Reticulum Kinase (PERK), ATF4 and CHOP (Wennemers et al., 2011b), three mediators of the unfolded protein response pathway which is activated upon the accumulation of misfolded proteins in the endoplasmic reticulum (ER) (Malhi and Kaufman, 2011). Depletion of TRIB3 mRNA in MDA-MB-231, a breast cancer cell line, resulted in the increased sensitivity of these cells to hypoxia (Wennemers et al., 2011b). However depletion of TRIB3 in the same breast cancer cell line has also been reported to increase hypoxia survival of the cells, this time after the cells had been irradiated (Wennemers et al., 2011a).

TRIB3 expression in oral tongue squamous cell carcinoma (OTSCC) is significantly higher in the tumour cells compared to adjacent non-cancerous tissue. Increased TRIB3 is closely correlated with tumour pathological T stage, lymph node metastasis and tumour recurrence in OTSCC. The ER-stress inducers thapsigargin and tunicmycin induced TRIB3 expression in OTSCC cell lines linking tumour cell survival to the ER-stress response (J. Zhang et al., 2011).

As in breast cancer patients colorectal cancer patients with high TRIB3 mRNA tumour expression had a significantly poorer prognosis for disease-free and overall survival compared to those with low TRIB3 expression. Analyses of colorectal cancer (CRC) samples has revealed that TRIB3 expression is higher in cancerous versus non-cancerous tissue. Metastasis of the cancer and patient survival is correlated with TRIB3 expression in the tumour samples and knock-down of TRIB3 expression in CRC cell lines resulted in a decrease in proliferation (Miyoshi et al., 2009).

In multiple myeloma cells TRIB3 and the expression of other CHOP induced ER stress related genes was induced by a combination of macrolide antibiotic and bortezomid. This drug combination lead to enhanced cytotoxicity in the myeloma cells compared to either drug alone indicating that the induction of the ER stress response has therapeutic potential in the treatment of multiple myeloma (Moriya et al., 2013).

1.5.2 Tribble and Obesity

Increases evidence has indicated a link between the Tribble genes and obesity. Dietary fat composition and obesity has been related to a number of diseases including cardiovascular disease and type II diabetes (Ros, 2003; Czernichow et al., 2010). Biopsies from obese patients and patients with type II diabetes show that they have significantly elevated levels TRIB3 in their muscles (Koh et al., 2013), TRIB1 has been reported to be a lipid- and myocardial infarction-associated gene (Burkhardt et al., 2010) and both TRIB2 and TRIB3 expression has been associated with the suppression of adipocyte differentiation (Naiki et al., 2007). Increased Trib3 expression has also been associated with the development of atherosclerosis in mice, a condition closely related to obesity (Berisha et al., 2013).

The expression of TRIB1 has been shown to regulate lipoprotein metabolism in the body. Increased Trib1 expression in mice, which is unusually high in human liver samples, reduced their levels of cholesterol and triglyceride (TG) levels (Burkhardt et al., 2010). Specifically Trib1 over-expression reduced plasmid levels of very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol. Trib1 deficient mice were also found to have

increased plasma cholesterol (predominantly VLDL and LDL cholesterol) and TG levels (predominantly VLDL and LDL TG). The expression of genes that are involved in fatty acid oxidation (Carnitine Palmitoyltransferase(Cpt)1a, Cpt2 and Acox1) is down-regulated in Trib1 deficient mice while the key lipogenic genes Acc1, Fasm and stearoyl-coA desaturase 1 (Scd1) are up-regulated (Burkhardt et al., 2010). Knock-down of Trib1 expression in Zebrafish also affects lipoprotein associated gene expression. Trib1 depletion results in the reduction of Fatty Acid Binding Protein 10a (fabp10a), a liver fatty acid binding protein, expression and increased susceptibility to liver damage (L. Y. Liu et al., 2013). While Trib1 haploinsufficiency was found to protect mice from high fat diet induced obesity (Ostertag et al., 2010). Exercise can affect TRIB1 levels in the skeletal muscle. TRIB1 shows decreased expression and the locus shows increased methylation after exercise in skeletal muscle cells (Nitert et al., 2012).

TRIB1 is also associated with the control of adipose tissue inflammation (Ostertag et al., 2010). TRIB1 expression (but not TRIB2 or TRIB3) is elevated in white adipose tissue (WAT) during in acute and chronic inflammation. The induction of TRIB1 expression in response to LPS was specific to whole WAT, mature adipocytes and the stroma vascular fraction (SVF) (but not CD11b+) macrophage-enriched cellular fractions (Ostertag et al., 2010).

TRIB1 is a lipid- and myocardial infarction-associated gene (Burkhardt et al., 2010). Single-nucleotide polymorphisms or SNPs associated with the TRIB1 locus have linked the TRIB1 gene to obesity, myocardial infarction and other illnesses. SNPs associated with TRIB1 have been determined to be both atheroprotective and to increased the risk of coronary artery disease (Teslovich et al., 2010; Waterworth et al., 2010). Specific SNPs such as the TRIB1 rs17321515 polymorphism has been

associated with severe hypertriglyceridema in European and Japanese populations (Kathiresan et al., 2008; Wang et al., 2008; Nakayama et al., 2009) as well as increase in total cholesterol and LDL-cholesterol levels in individuals Asian Malays (Tai et al., 2009) and with serum HDL and LDL cholesterol in Mulao and Han populations and triglyceride levels in the Han population (Aung et al., 2011; Lan et al., 2013). Other SNPs have associated mutations in the TRIB1 locus with increased triglyceride, LDL-cholesterol and HDL-cholesterol levels (Willer et al., 2008; Edmondson et al., 2011; Turner et al., 2011; Z. Zhang et al., 2011) and with liver enzymes in plasma (an indicator of liver disease) (Chambers et al., 2011). TRIB1 polymorphisms have even been associated with sleep duration, indicating a possible link between sleep and metabolism (Ollila et al., 2012).

Trib3 expression also affects lipid metabolism in mice. The liver of the fatty liver dystrophic mouse contains elevated levels of Trib3 (Klingenspor et al., 1999). Transgenic mice specifically over-expressing TRIB3 in their adipose tissue were found to be leaner than their wild-type counterparts. Trib3 protected the mice from diet induce obesity by enhancing rates of fatty acid oxidation. TRIB3 was found to associate with and inhibit the activity of Acetyl-CoA carboxylase (ACC), the rate limiting enzyme in fatty acid synthesis (Ruderman et al., 2003), by associating with and recruiting the E3 ubiquitin ligase COP1, leading to the COP1 mediated ubiquitination and subsequent degradation of Acetyl-CoA Carboxylase (ACC) stimulating lipolysis (Qi et al., 2006).

TRIB3 expression in hepatocytes is differentially regulated by saturated and unsaturated fatty acids (Geng et al., 2013). The induction of TRIB3 by saturated fatty acid was found to occur via the ATF4/CHOP-dependent ER stress pathway. Introducing unsaturated fatty acids to hepatocytes prevented this induction as well as

increased Trib3 levels mice on a saturated fatty acid diet had higher fasting blood glucose, fasting insulin, postprandial hyperinsulinaemia and insulin secretion levels compared to mice feed on the high fat diet composed of unsaturated fatty acids. Therefore the composition of the fat in diet can affect hepatic TRIB3 induction, ER stress and the insulin response pathways (Geng et al., 2013).

1.5.3 Tribble and Diabetes

Not only have the Tribble genes been associated with obesity, increasing evidence has also associated these genes with the development of type II diabetes mellitus linking obesity and the development of insulin resistance. Investigations into the link between the Tribble and diabetes have primarily linked TRIB3 expression to this disease.

Insulin signalling is responsible for the promotion of the uptake of glucose into the cell, particularly muscle cells and the suppression of glucose production in the liver. Resistance to insulin signalling is one of the hallmarks of type II diabetes. Binding of insulin to its receptor triggers a phospholipid-dependent kinase cascade. This cascade culminates with the phosphorylation of the Ser-Thr kinase AKT (Brazil and Hemmings, 2001; Schinner et al., 2005). Both TRIB2 and TRIB3 have been found to interact and inhibit phosphorylation of AKT thereby inhibiting AKT activation in the cell during adipocyte differentiation (Naiki et al., 2007), potentially linking both these genes to the control of insulin signalling in the cell.

Compelling research has emerged that suggests that increased TRIB3 expression can lead to the development of type II diabetes mellitus and links obesity to the development of insulin resistance. Patients with diabetes or obesity have increased levels of TRIB3 expression in their skeletal muscles (Koh et al., 2013). TRIB3

expression is induced in the liver by insulin expression, by ER stress, and by fasting and Trib3 is highly expressed in the liver of diabetic mice (Du et al., 2003; Matsumoto et al., 2006; Ding et al., 2008; Du and Ding, 2009; Koh et al., 2013). TRIB3 is then found to impair insulin signalling and insulin-stimulated glucose uptake in the cell (Koh et al., 2013). Trib3 expression is also increased in the kidneys and in the testis of diabetic mice indicating that increased Trib3 expression is associated with insulin resistance in other organs the body (Morse et al., 2010; Zhao et al., 2012).

Trib3 knock-out mice ($Trib3^{-/-}$ mice) show no difference in serum glucose, insulin, or lipid levels; glucose or insulin tolerance; or energy metabolism compared to their wild-type litter mates (Okamoto et al., 2007; Koh et al., 2013). This indicates that the role played by Trib3 in insulin signalling is non-redundant. However these $Trib3^{-/-}$ mice did show deficiencies in the ER stress-induced insulin-stimulated glucose uptake. These mice were found to be protected from diet-induced insulin resistance brought about by a high fat diet. (Koh et al., 2013).

As well as insulin expression both peroxisome proliferator-activated (PPAR)-γ coactivator-1 or PGC-1 and chronic ethanol consummation have all been shown to induce Trib3 expression in the liver (Koo et al., 2004; He et al., 2006). PGC-1, a nuclear hormone receptor co activator, is induced in the mouse model of diabetes where is seems to contribute to insulin resistance (Herzig et al., 2001; Yoon et al., 2001). Chronic ethanol consummation can result in insulin resistance and type II diabetes as well as alcoholic liver disease (Wei et al., 2000; Kao et al., 2001).

While TRIB1 and TRIB2 expression has not been associated with the development of diabetes these genes may play a role in the development of arteriosclerotic

plaques associated with diabetes. Both TRIB1 and TRIB2 expression has also been found to be significantly higher both in stable and unstable plaques and up-regulation of Trib2 expression induced by miR-98 has been observed in the early lesion of the large arteries of rats with type-2 diabetes (Deng et al., 2009; Xie et al., 2012). Inflammation and the immune affecter mechanism is associated with the development of atherosclerosis (Libby et al., 2013). Elevated TRIB1 expression in the arterial plaques is associated with the presence of macrophage cells, while TRIB2 expression was high enough not to be associated with macrophage expression alone. Instead increased TRIB2 expression was deemed to be associated with the unstable plaque region itself and was associated with a decrease in IL-10 mRNA levels, an arthereoprotective cytokine (Deng et al., 2009).

1.5.4 Tribble and Autoimmune Disorders

Of the three Tribble genes only TRIB2 has been linked to autoimmune disorders. Anti-TRIB2 autoantibodies have been associated with narcolepsy in both Caucasians (Kawashima et al., 2010) and Japanese patients (Toyoda et al., 2010) as well as in narcolepsy patients who also suffer cataplexy (Cvetkovic-Lopes et al., 2010; Kawashima et al., 2010). Further investigation has revealed that anti-Trib2 autoantibodies cause orexin neuron loss and sleep attacks in mice (Katzav et al., 2013). TRIB2 has also been identified as an autoantigen in autoimmune uveitis (Zhang et al., 2005).

1.6 Tribble and Leukaemia

As the Tribble proteins are considered to be signal modulators involved in the control and transduction of extra and intracellular signals in the cell, dysregulation of Tribble expression or activation leads to perturbations in these cellular response pathways potentially resulting in disease states. In 2006 Trib2 was identified as an oncogene that causes acute myeloid leukaemia (AML) (Keeshan et al., 2006). Following shortly came a link between Trib1 and leukaemia when Trib1 was found to cooperate with Homeobox(Hox)a9 and Meis1 to significantly accelerate the onset of myeloid leukaemogenesis in mice (Jin et al., 2007).

1.6.1 Introduction to Leukaemia

According to the National Cancer Registry Ireland leukaemia is the 11th most commonly diagnosed cancer in women and the 9th most commonly diagnosed cancer in men in Ireland. In 2010 leukaemia was responsible for 2.6% of Irish cancer deaths (National Cancer Registry Ireland (NCRI), 2013). Leukaemia is a clonal disease that arises from genetic and epigenetic alterations in the haematopoietic steam or progenitor cells. These genetic defects compel unrestrained proliferation and disrupt normal myeloid or lymphoid differentiation programmes ultimately leading to bone marrow failure. Leukaemia is initially classified as a myeloid or lymphoid leukaemia depending on which haematopoietic cell lineage is disrupted. Mutations in the very early and uncommitted haematopoietic stem cells can also occur which sometimes leads to leukaemia with a mixture of myeloid and lymphoid characteristics (Brown et al., 2012; Hasserjian, 2013).

Leukaemia can then be further classified as either the acute or chronic form of the disease. The acute leukaemias are clinically defined by a more rapid disease which progresses over a number of weeks and months ultimately leading to bone marrow failure. Chronic leukaemia is a less aggressive form of the disease characterised by a prolonged clinical course even if, in some cases, left untreated (Brown et al., 2012).

Initially leukaemia is classified as acute or chronic and as either myeloid or lymphoid depending on which progenitor cell lineage is disrupted (table 1.1). Acute myeloid leukaemia (AML) is probably the most heterogeneous of all leukaemeias, and is further classified on the basis of cytogenetic (present in 50-60% of AML cases) and molecular as well as morphological criteria. Based on these classification systems a patient diagnosed with AML can be stratified into groups with favourable, intermediate or adverse prognosis (table 1.2) (Breems et al., 2008; Brown et al., 2012). Acute lymphoblastic leukaemia (ALL) is also further defined on the basis of cytogenetic and molecular abnormalities, as well as on immunophenotype criteria (Brown et al., 2012) (table 1.2).

Chronic lymphoid leukaemia (CLL) is defined by the aberrant proliferation and accumulation of mature B-cells in the body. Proliferation is clonal in nature and occurs in the blood, bone marrow, lymph nodes, and spleen. In western countries CLL is one of the most common types of leukaemia (Gibson et al., 2013; Hallek, 2013).

Chronic myeloid leukaemia (CML) is the best characterized stem cell disease defined by the excessive production of mature myeloid cells that are both morphologically abnormal and dysfunctional. The single biggest risk factor for developing AML is radiation exposure. In excess of 95% of patients diagnosed with

CML posses a chromosomal translocation known as the Philadelphia chromosome (Ph). This chromosomal abnormality is formed by the reciprocal translocation of chromosomes 9 and 22 which gives rise to the *BCR-ABL1* fusion gene. This gene encodes a chimeric protein with enhanced tyrosine kinase activity (ABL is a weak tyrosine kinase) that is sufficient for leukaemogenesis.

CML progresses in two distinct phases. First there is the chronic phase of the disease that lasts on average 4 to 5 years. This is followed by an aggressive phase known as the blast phase of the disease that resembles acute myeloid or lymphoid leukaemia. Though rare CML was the first malignancy in which a recurrent cytogenetic abnormality was identified. This makes it an extremely important disease in advancing the understanding of stem cell disorders and the clonal nature of leukaemia. The introduction of imatinib mesylate, a tyrosine kinase inhibitor with potent activity against BCR-ABL, revolutionised CML treatment. Imatinib therapy showed 85% overall survival in an 8 year follow up trial, though approximately 30% of patients will discontinue use due to development of resistance or side-effects (Krämer et al., 2001; Deininger et al., 2009; Gibson et al., 2013; Rana et al., 2013). Unfortunately other forms of leukaemia remain on standard therapies and lag behind the success of imatinib treatment of CML.

Myelodysplastic syndromes (MDS) are clonal myeloid disorders of haematopoiesis associated with progressive peripheral blood cytopenias and ineffective myelopoiesis. The affected haematopoietic progenitor cells are frequently found to posses genetic aberrations. The cytogenetics of the abnormal cells plays an important role in the prognostic determination of MDS. MDS can evolve into full blown AML but even without this progression of the diseases there is an increased risk of death

due to bone marrow failure (Gibson et al., 2013; Kulasekararaj et al., 2013; Natelson and Pyatt, 2013).

Approximately 3-5% of acute leukaemia either co-express different lineage markers (bi-phenotypic) or are characterised by the appearance of leukemic cells of different lineages (bi-lineal). These types of leukaemia are termed mixed lineage leukaemia and are often of a myeloid–B or myeloid–T phenotype or rarely of a T-B phenotype. T-Acute Lymphoblastic Leukaemia (T-ALL) or B-ALL with myeloid markers and AML with lymphoid markers has also been observed. These leukaemias are believed to be derived from progenitor cells with both myeloid and lymphoid potential such as the LMPP. Progenitor cells with both myeloid and lymphoid potential that undergo transformation could maintain both the myeloid and lymphoid programs resulting in mixed lineage leukaemia (Weir et al., 2007; Bene, 2009; Rubnitz et al., 2009; Kawamoto et al., 2010).

Leukaemia Classification		
Acute versus Chronic	**Progenitor Cell Lineage Affected**	**Leukaemia Type**
Acute	Myeloid	Acute Myeloid Leukaemia
Acute	Lymphoid	Acute lymphoblastic leukaemia
Chronic	Myeloid	Chronic myeloid leukaemia
Chronic	Lymphoid (mature B-cells)	Chronic lymphoid leukaemia

Table 1.I: Classification of leukaemia based on disrupted cell type and speed of disease progression. Information obtained from Brown et al., 2012 and Hasserjian, 2013.

Sub classification of AML		Sub classification of ALL
Prognosis	**Cytogenetic Classification**	**Based on Cytogenetic, Molecular and Immunophenotype Criteria**
Favourable	AML with t(15;17) or Acute promyelocytic leukaemia	B-ALL not otherwise specified
	AML with t(8;21) AML with inv(16) AML with t(16;16)	B-ALL with recurrent genetic abnormalities ALL with t(9;22) ALL with t(v;11q23) ALL with t(12;21) ALL with t(5;14) ALL with t(1;19) ALL with Hyperdiploid Karyotype ALL with Hypodiploid Karyotype T-ALL Mature B-ALL
Intermediate	Normal Karyotype (can be further classified based on genetic abnormalities such as FLT3-ITD mutations or CEBPA mutation) Trisomy 8 AML with t(9;11)	
Adverse	Complex Karyotype Monosomal Karyotype -5 or del(5q) -7 Abnormalities of 17p 11q23 abnormalities	

Table 1.II: Sub classification of both AML and ALL based on cytogenetic, molecular and/or immunophenotype criteria. The AML samples are also sorted into prognostic groups based on their sub classification. Information was derived from Brown et al., 2012.

1.6.2 The Tribble and Leukaemogenesis

Both Trib1 and Trib2 are oncogenes involved in the induction of murine AML (Keeshan et al., 2006; Jin et al., 2007). Mice reconstituted with bone marrow cells transduced with either Trib1 or Trib2 (but not Trib3) develop transplantable AML (Keeshan et al., 2006; Dedhia et al., 2010). Trib2 was identified as a Notch1 target that promotes monocyte and inhibits granulocytic differentiation in mice thereby perturbing myeloid development *in vivo* (Keeshan et al., 2006). Trib1 and Trib2 were found to degrade full length C/EBPα protein inhibiting myeloid differentiation (Keeshan et al., 2006; Dedhia et al., 2010). Both Trib1 and Trib2 transduced cells were found to exhibit growth advantage *in vitro* as Trib1 and Trib2 conveyed serial plating potential to transduced murine bone marrow cells (Dedhia et al., 2010). Trib2 transduced cells were also able to establish factor dependent long term myeloid progenitor cell lines (Keeshan et al., 2006).

Trib2 drives the induction of murine AML with a robust and short latency. However the leukaemia that Trib2 gives rise to are clonal and this suggests that a secondary hit is occurring in order to give rise to overt AML (Keeshan et al., 2006). Both Trib1 and Trib2 can cooperate with other genes to induce murine AML. Trib1 was first discovered as a gene that cooperates with Meis1 and HoxA9 to induce murine AML (Jin et al., 2007). Subsequently it was discovered that Trib2 could cooperate with HoxA9 alone in the induction of myeloid leukaemia (Keeshan et al., 2008). Trib2 has also been separately linked to Meis1 leukaemogenic activity (Argiropoulos et al., 2008), Trib2 was shown to complement the induction of AML by ND13 (a HOX fusion protein of Nucleoporin 98kDa (NuP98) and HOXD13). Meis1 is known to collaborate with a multitude of Hox and NuP-HOX fusion proteins to accelerate the

onset of AML though the molecular mechanism by which it achieves this is still unknown (Argiropoulos et al., 2008).

It is thought that Trib1 and Trib2 leukaemogenesis is linked to the ability of these proteins to degrade the myeloid transcription factor C/EBPα. Both Trib1 and Trib2 were found to interact with and degrade the C/EBPα protein (Keeshan et al., 2006; Dedhia et al., 2010). Trib1 and Trib2 are thought to induce this degradation by complexing with COP1, an ubiquitin ligase, and the ability of both Trib1 and Trib2 to degrade C/EBPα is dependent on the presence of a conserved COP1 binding domain found in both these proteins. Mutants of both Trib1 or Trib2 that lack this COP1 domain are unable to induce murine AML indicating that this binding site is crucial for their leukemic activity (Keeshan et al., 2010; Yokoyama et al., 2010). Most recently Trib1 was shown to cooperate with COP1 in the induction of murine AML (Yoshida et al., 2013). Trib3, unlike Trib1 and Trib2, cannot induce murine AML. Nor can it convey serial plating potential to transduced murine bone marrow cells, degrade C/EBPα or inhibit myeloid differentiation (Dedhia et al., 2010).

In recent years TRIB1 and TRIB2 expression has been linked to other forms of leukaemia. TRIB1 expression is abnormally increased in Janus Kinase 2(JAK2)V617F-neagtive essential thrombocythenia compared to JAK2V617F-positive patient samples (Puigdecanet et al., 2008). TRIB1 expression has also been found to be increased in CML leukaemia patients with variant t(9;22) compared to CML patients with classic t(9;22) tranlocations (Albano et al., 2013). High TRIB2 expression has been associated with poor prognosis in CLL (Johansson et al., 2010) and PITX, which is activated by the del(5)(q31) in T-ALL, was found to activate the expression of the TRIB2 in jurkat cells, as well as other genes involved in T-cell development (Nagel et al., 2011).

Keeshan et al. (2006) noted that a subset of human AML patients exhibiting C/EBPα defects possessed elevated TRIB2 expression (the existence of a subset of human AML patients samples expressing high TRIB2 has also been noted by Stirewalt et al. (2008)). This discrete subset of AML patients were discovered to have a distinct gene expression profile that included silenced C/EBPα expression, expression of T-lymphoid genes (including CD7, CD3 and T Cell Receptor Delta Locus (TRD@)) and elevated Notch1 expression. Three of the AML patient in this cohort possessed activating Notch1 mutations, and indeed it was shown that TRIB2 is a direct target of Notch1 (Wouters et al., 2007). Notch1 mutations are the most common form of mutation observed in T-ALL (activating mutations are reported to occur in 34-71% of all adult and paediatric T-ALL patients) (Weng et al., 2004; Breit et al., 2006; Kraszewska et al., 2012). Here a distinct subset of AML with a T-ALL like signiture is linked to aberrant TRIB2 expression.

Trib1 over-expression in murine bone marrow was also found to significantly enhance the activation of the MAPK signalling pathway in the leukemic cell (Jin et al., 2007). MEK1 binding was found to be vital for the induction of leukaemia by Trib1. Trib1 was found to link the MAPK signalling pathway and C/EBPα degradation in leukaemia as MEK1 inhibition in the cell could also to inhibit Trib1 mediated degradation of C/EBPα (Yokoyama et al., 2010).

So far, one instance of a TRIB1 mutation in a human leukaemia patient has been reported. In 2012 Yokoyama et al. reported the existence of a Down syndrome related acute megakaryocytic leukaemia patient with an R107L gain of function mutation in TRIB1. The investigators concluded that the mutation was somatic and occurred in the haematopoietic stem cell. Over-expression of TRIB1 with the R107L mutation, which is located in the psuedokinase domain of TRIB1, was found to

induce a more aggressive acute myeloid leukaemia (AML) with significantly shorter survival time in mice compared to wild-type. This mutant enhanced ERK phosphorylation more extensively than wild-type TRIB1 in the AML cells.

Analyses of AML patient samples with double minutes (dmin) consisting of an amplified segment from chromosomal band 8q24 revealed that while this amplified band always contains the MYC the expression of this gene is silenced. Instead a number of these patient samples over-express TRIB1, which is also located in the 8q24 chromosomal band (Storlazzi et al., 2006).

Dysregulated TRIB1 expression due to a chromosomal amplification was reported in an AML patient with Li-Fraumeni-like Syndrome (LFLS), a condition that predisposes and individual to cancer. The AML cells of the patient had mutated Tumor Protein p53 (TP53), a tumour suppressor which is involved in the maintenance of genetic stability, mutations of which are associated the LFLS. Two amplicons were also identified. One, 8q24.2, was found to target TRIB1 as well as Myc and PYT1. TRIB1 was believed to be the target gene inducing AML as it was the only gene that showed increased expression in the AML cells (Sugawara et al., 2011).

While Trib3 cannot induce AML in mice (Dedhia et al., 2010) it may still play a role in leukaemia. Analyses of MDS patients with normal karyotype using SNP array data has indicated that TRIB3, located in 20p uniparental disomies (UDP), may be a potential candidate gene contributing to MDS (Merkerova et al., 2012) and TRIB3 expression has been found to be up-regulated in samples from AML patient with good prognosis (Park et al., 2007). However to date there is no evidence to directly tie TRIB3 expression to the development of either murine or human leukaemia.

In 2010 Gilby et al. reported that both TRIB1 and TRIB2 are tumour suppressors found to be down-regulated in AML. Of the five leukaemia cell lines screened (Me-1, Kasumi-6, U937, Human Erythroleukemia cell line (HEL) and K562) knock-down of TRIB1 or TRIB2 expression in only the Me-1 cells, lead to an increase in cellular proliferation. Over-expression of TRIB1 or TRIB2 was also found to increase apoptosis in the Me-1 cell line. TRIB1 and TRIB2 were found to decrease JNK and B-Cell Lymphoma 2 (Bcl-2) phosphorylation in Me-1 cells. Bcl-2 is a key anti-apoptotic protein phosphorylation of which is key to its function, JNK has been shown to phosphorylate Bcl-2 (Ito et al., 1997; Ruvolo et al., 1998; Deng et al., 2000). The authors postulate that inhibition of JNK phosphorylation by TRIB1 or 2 lead to a decrease in Bcl-2 phosphorylation leading to a decrease in its anti-apoptotic activity. However the overwhelming evidence to date indicates that both TRIB1 and TRIB2 are oncogenes that cause AML and while median TRIB2 expression is down-regulated in AML compared to normal bone marrow a small subset of patients show abnormally high levels of TRIB2 expression (Keeshan et al., 2006; Jin et al., 2007; Stirewalt et al., 2008; Dedhia et al., 2010). Apart from Me-1 cells TRIB2 has been found to induce apoptosis via a TRIB2-Mcl-1 dependant pathway in TF-1 cells upon survival factor withdrawal (Lin et al., 2007), but over-expression of TRIB2 in lung cancer cells has been associated with a reduction of apoptosis (Grandinetti et al., 2011) indicating that TRIB2 induced apoptosis is cell type and context specific. TRIB1 and TRIB2 may be activating a pro-apoptotic pathway in the Me-1 cell line that is mutated or suppressed in the other leukemic cell lines. This would lead to pro-apoptotic instead of oncogenic activity of TRIB2 or TRIB1, as suggested by the fact that knock-down of TRIB2 or TRIB1 expression enhanced the proliferation of the

Me-1 cell line but not the proliferation of Kasumi-6, U937, HEL or K562 leukemic cell lines (Gilby et al., 2010).

1.7 Aims of Project

The aim of this project was to study the regulation of the Tribble family members both in normal haematopoiesis and in leukaemogenesis. The Tribble family of genes play a key role as modulators of a variety of cellular processes including differentiation, proliferation and apoptosis. The dysregulation of these vital cellular processes can result in the development of abnormal haematopoietic cells and aid their survival and proliferation ultimately leading to the development of leukaemia. As the Tribble genes are involved in the regulation and coordination of these processes it is not surprising that members of this gene family have been are implicated in leukaemogenesis.

The aim of this work was to dissect the regulation of the Tribble genes, particularly Trib1 and Trib2 which both induce murine AML. Leukaemia offers an excellent opportunity to study the molecular events that lead to cancer as it is a clonal disease that arises from genetic and epigenetic alterations in the haematopoietic steam or progenitor cells. Here we investigate the association of the Tribble genes with the different subtypes of leukaemia and the different cells of haematopoiesis. We also aim to identify pathways and transcription factors that may play a functional role in the regulation of the Tribble genes in both the normal cells of haematopoiesis and in the leukemic cell using both bioinformatic and wet lab methods.

1.8 Hypothesis

Can mining leukaemia and haematopoietic microarray datasets identify pathways associated with and transcription factors involved in the regulation of TRIB1 and TRIB2 either in leukemic or haematopoietic cells?

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 General Chemicals and Reagents

All salts and reagents were purchased from Sigma-Aldrich (Dublin, Ireland) unless otherwise stated. Bio-Rad Dual Colour Precision Plus Protein Prestained Standards were purchased through Alpha Technologies (Wicklow, Ireland). DNA ladders 100 base pairs (bp) and 1 Kilobase (kb), all restriction enzymes and DNA ligaise were purchased through NEB ISIS (Wicklow, Ireland). Active Motif ChIP-IT Express Chromatin Immunoprecipitation Kits were purchased through MyBio (Kilkenny, Ireland). KAPA SYBR® FAST Universal 2X quantitative PCR (qPCR) Master Mix (5 ml) was purchased through Anachem (Bedfordshire, United Kingdom). SensiMix SYBR No-ROX Kit was purchased from Bioline through MyBio (Kilkenny, Ireland). Glycylglycine was purchased from Calbiochem through Merck Millipore (Cork, Ireland).

Turbofect transfection reagent was purchased from Thermo Scientific Pierce through Fisher Scientific Ireland (Dublin, Ireland).

D-Luciferin (potassium salt) was purchased from Gold Biotechnology (St. Louis, Missouri, USA).

Anti-Rabbit and Anti-Mouse IgG HRP (Horseradish Peroxidase) Linked Whole Ab was purchased from GE Healthcare through Fisher Scientific (Dublin, Ireland).

Roswell Park Memorial Institute (RPMI) 1640 medium and RPMI 1640 medium (no phenol red) were purchased from Invitrogen through Life Technologies Bio-Sciences (Dublin, Ireland).

RNaseOUT™ Recombinant Ribonuclease Inhibitor, SuperScript® III Reverse Transcriptase and dNTPs for complementary DNA (cDNA) Probe Synthesis were purchased from Invitrogen through Life Technologies Bio-Sciences (Dublin, Ireland).

Plasmocin purchased from Invivogen (Toulouse, France).

MycoAlert Mycoplasma Detection Kit purchased from Lonza (Dublin, Ireland).

pSTBlue-1 AccepTor Vector Kit purchased from Merck Millipore (Cork, Ireland).

Pierce Enhanced Chemiluminescence (ECL) Western Blotting Substrate , Mouse HRP, Rabbit HRP, Dynamo cDNA Synthesis Kit, Supersignal west pico chemiluminescent substrate, Dnase I, Restore Western Blot Stripping Buffer, Cl-Xposure film and SuperSignal West Femto Substrate Trial Kit were purchased from Thermo Scientific Pierce through the Medical Supply Company Ltd. (Dublin Ireland) or through Reagacon (Clare, Ireland).

Seal absolute optically clear for qPCR were purchased from Thermo Scientific Pierce through Fisher Scientific Ireland (Dublin, Ireland) and Microplates for PCR-96 well purchased from Reagacon (Clare, Ireland).

Fast Start High Fidelity Polymerase Chain Reaction (PCR) System, Fast Start Taq DNA Polymerase, G-C Rich PCR System LightCycler® 480 Sealing Foil and PCR Nucleotide Mix were purchased from Roche (Dublin, Ireland).

Pure Yield Plasmid Midiprep System, Passive Lysis Buffer and Pfu DNA Polymerase were purchased from Promega through the Medical Supply Company Ltd. (Dublin Ireland).

Attractene Transfection reagent was purchased from Qiagen (West Sussex, England).

2.1.2 Molecular Biology Reagents

Primers were ordered from and synthesised by Eurofins MWG Operon (Ebersberg, Germany).

Fast Start High Fidelity PCR System, Fast Start Taq DNA Polymerase, G-C Rich PCR System LightCycler® 480 Sealing Foil and PCR Nucleotide Mix were purchased from Roche (Dublin, Ireland).

Rnase-free Dnase set, Random Hexamers, RNeasy Mini Kit, Qiashredder and QIAquick PCR Purification Kit from Qiagen (West Sussex, England).

SafeView Nucleic Acid Stain was purchased from NBS Biologicals Ltd. (Cambridgeshire, England).

2.1.3 Plasmid Sources

pLPC E2F1 and pLPC E2F1 E132 were a kind gift from Dr. Kevin Ryan (Beatson Institute, Glasgow, UK).

pcDNA3 E2F1, pcDNA3 E2F4 and pcDNA3 E2F5 were a kind gift from Dr Shaun Thomas (King's College London, UK).

pcDNA3 E2F3 was a kind gift from Dr. John A. Pulikan (Massachusetts Mediaca School, USA)

MigRI PU.1 and MigRI Hry were a kind gift from Dr. Warren S. Pear (University of Pennsylvania, USA).

Trib2 Promoter (T2P) 500 and T2P 2.6 cloned by Karen Keeshan.

The following plasmids were used:

Name	Construct	Backbone
pGL3 Basic	Empty	pGL3 Basic
T2P 500	T2P 500	pGL3 Basic
T2P 2.6	T2P 2.6	pGL3 Basic
T2P 800	T2P 800	pGL3 Basic
T2P 927	T2P 927	pGL3 Basic
T2P 963	T2P 963	pGL3 Basic
T2P Mut. A	T2P Mut. A	pGL3 Basic
T2P Mut. B	T2P Mut. B	pGL3 Basic
T2P Mut. C	T2P Mut. C	pGL3 Basic
T2P DM	T2P DM	pGL3 Basic
pRL-TK	*Renilla*	pRL
CMV E2F1	E2F1	CMV
MigRI HRY	HRY	MigRI
MigRI C/EBPα	C/EBPα	MigRI
MigRI PU.1	PU.1	MigRI
pCGNHAM MYC	c-MYC	pCGNHAM
MigRI C/EBPβ	C/EBPβ	MigRI
pcDNA3	Empty	pcDNA3
pcDNA3 E2F1	E2F1	pcDNA3
pcDNA3 E2F3	E2F3	pcDNA3
pcDNA3 E2F4	E2F4	pcDNA3
pcDNA3 E2F5	E2F5	pcDNA3
pLPC E2F1	E2F1	pLPC
pLPC E132	E2F1 E132	pLPC

Table 2.I: Table of all plasmids used in experimental work for this thesis. Name refers to the name of the plasmid construct, construct is the cloned gene inserted into the plasmid. Backbone refers to the backbone of the plasmid into which the relevant coned gene was inserted. Maps of all backbones used can be found in appendix C.

2.1.4 Antibodies

Rabbit polyclonal E2F1 (c-20),E2F2 (c-20), E2F3 (N-20), E2F4 (c-20) and E2F5 (E-19) antibodies, SV40 T Ag and Trib2 mouse monoclonal antibodies were all sourced from Santa Cruz through Fannin Limited (Dublin, Ireland). Actin mouse monoclonal antibody was sourced from Sigma-Aldrich (Arklow, Ireland).

CD11b (apc) and CD 15 (gfp) were sourced from eBiosciences (Hatfield, United Kingdom).

2.1.5 Cell lines

Murine 32D cells, human U937 cells, human NB4 cells and Human Embryonic Kidney 293T (HEK293T) cells from lab stocks were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) foetal bovine serum (FBS), 1% (v/v) L-glutamine, and antibiotics (50units/ml penicillin and 50µg/ml streptomycin).

Murine 3T3 cells from lab stocks Dulbecco's modified Eagle's medium (DMEM-21) containing 10% (v/v) cosmic serum, 1% (v/v) L-glutamine, and antibiotics (50units/ml penicillin and 50µg/ml streptomycin).

Fibroblasts derived from E2F1-deficient or wild-type mouse embryos were a kind gift from Dr. Lili Yamasaki (Columbia University, USA).

All murine embryonic fibroblast (MEF) cell lines were grown in DMEM, containing 10% (v/v) foetal bovine serum, 1% (v/v) L-glutamine, and antibiotics (50units/ml penicillin and 50µg/ml streptomycin).

The murine RAW264 cell line were a kind gift from Dr. Ruaidhrí Carmody (Glasgow, UK) and were grown in DMEM, containing 10% (v/v) FBS, 1% (v/v) L-glutamine, and antibiotics (50units/ml penicillin and 50µg/ml streptomycin).

The murine 32D cell line from lab stocks were cultured in Iscove modified Dulbecco medium (IMDM) containing 10% (v/v) FBS and 10% (v/v) WEHI-conditioned media, 1% (v/v) L-glutamine, and antibiotics (50units/ml penicillin and 50µg/ml streptomycin).

The human K562, Molm-13 and Thp-1 cell lines were kind gifts from Prof. Thomas G. Cotter (Cork, Ireland). Cells were cultured in we RPMI 1640 containing 10% (v/v) foetal bovine serum, 1% (v/v) L-glutamine, and antibiotics (50units/ml penicillin and 50µg/ml streptomycin).

U937 human cell lines from lab stocks were cultured in RPMI 1640 medium containing 10% (v/v) FBS, 1% (v/v) L-glutamine, and antibiotics (50units/ml penicillin and 50µg/ml streptomycin).

HL-60 cells from lab stocks were grown in IMDM containing 10% (v/v) FBS, 1% (v/v) L-glutamine, and antibiotics (50units/ml penicillin and 50µg/ml streptomycin).

K562-C/EBPα-p42-ER, K562-C/EBPα-p30-ER, K562-C/EBPα-BRM2-ER, and K562-ER cells were a kind gift from Dr. Daniel G. Tenen (Boston, MA, USA). These cell lines maintained in RPMI 1640 without phenol red supplemented with 10% charcoal-treated FBS, 1% penicillin-streptomycin, and 2 µg/mL puromycin.

2.1.6 Bacterial Strains and Media used

Lab stocks of the *E. coli* DH5α strain were grown in pre-made Luria-Bertani (LB) broth and LB agar purchased Sigma-Aldrich (Arklow, Ireland).

2.2 Methods

2.2.1 Molecular Biology

2.2.1.1 Sequencing

DNA sequencing of all plasmids and clones was performed by GATC Biotech (Konstanz, Germany). Sequence alignment and analyses was performed using BLAST (Altschul et al., 1997).

2.2.1.2 Competent cell preparation and transformation

DH5α bacteria cells were streaked for single colonies and grown on an LB plate overnight at 37°C, a single colony was picked (or sample of an aliquot DH5α glycerol stock (single colony culture stocks)) and grown in 5ml of LB medium (no antibiotic) overnight at 37°C. 1. 5ml of this culture was transferred into 350ml LB broth (no antibiotics) and cultured at 37^{o}C in a shaking incubator. Once cell density reached OD_{600} 0.35-0.45 the culture was transferred into 6 x 50 ml tubes and placed on ice for 10 minutes. Cells were harvested by centrifugation of cells at 3000 revolutions per minute (rpm) in an Eppindorf 5415 R Centrifuge for 10 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 5 ml of 0.1 M $CaCl_2$ and placed on ice for 10 minutes. Samples were centrifuged at 3000 rpm in an Eppindorf 5415 R Centrifuge for 10 minutes at 4^{o}C. Each pellet was then

resuspended in 1 ml 0.1 M CaCl2 / 14% glycerol. 100 µl aliquots were placed in pre-cooled 1.5 ml eppendorfs and stored at -80°C.

2.2.1.3 Transformation of DH5α competent cells

Competent cells were removed from -80°C storage and rapidly thawed. 3 µl of competent cells was transferred into a fresh eppendorf per transformation. Each aliquot was incubated on ice for 10 minutes. 1 µl of the plasmid DNA (stock concentration 10-50 ng in total) was added to the competent cells and mixed gently by flicking. The sample was then incubated at 37°C for 1 minute. 100 µl of LB broth (no antibiotics) was then added to the sample at room temperature. The sample was then immediately plated onto an LB agar plate containing an appropriate antibiotic (50 µg/ml ampicillin or 50 µg/ml kanamycin) and incubated at 37°C overnight.

If transforming ligation reactions the protocol was modified as follows. 100 µl competent cells were removed from -80°C storage and placed on ice for 10 minutes. Ligation reaction DNA was then added to the cells and the sample was gentle mixed by flicking and placed back on ice for between 5 and 30 minutes. Sample was then placed in a 37°C waterbath for 5 minutes. At room temperature 250 µl of LB broth (no antibiotic) was added to the sample which was then placed in a 37°C waterbath for 30 minutes to 1 hour. The sample was then plated onto an LB agar plate containing an appropriate antibiotic (50µg/ml ampicillin or 50µg/ml kanamycin) and incubated at 37°C overnight.

2.2.1.4 Mini Preparation of Plasmids

Colony of interest was picked and grown overnight in 3ml of LB broth containing an appropriate antibiotic (50 µg/ml ampicillin or 50 µg/ml kanamycin) at 37 °C in a

shaking incubator. 1ml of supernatant was poured into a microcentifuge tube and spun for approximately 30 seconds at 16,100 relative centrifugal force (rcf). Supernatant was discarded and the pellet was resuspended in 50µl of EZ buffer (Easy buffer (EZ) buffer recipe; 10mM Tris.cl pH 8.0, 1mM Ethylenediaminetetraacetic Acid (EDTA), 15% w/v sucrose, 2mg/ml lysozyme, 0.2mg/ml pancreatic ribonuclease (RNase) (boiled for 30 minutes before use in order to kill Dnase), 0.1mg/ml Bovine Serum Albumin (BSA). Sample was vortexed for 5 minutes at full speed. Samples were then boiled for exactly 60 seconds and then immediately placed in ice for 60 seconds. The sample was then spun at full speed in a centrifuge for 25 seconds to pellet debris. The supernatant was transferred to a fresh eppendorf. Supernatant were then stored at -20^{o}C.

This method was only used to isolate plasmids for screening purposes. For transfection and sequencing plasmids were mini prepped using the Qiagen QIAprep Spin Miniprep Kit.

2.2.1.5 Plasmid DNA Restriction Digest

All restriction digests were performed according to NEB ISIS guidelines (https://www.neb.com/). To digest DNA between 500 ng – 1 µg of DNA was incubated with 2 µl of 10x Buffer, 2 µl of BSA if necessary and 0.5 µl of the restriction enzyme. The reaction mixture was made up to 20 µl with dH_2O and incubated for maximum 2 hours at 37^{o}C before being viewed by DNA Gel Electrophoresis.

2.2.1.6 DNA Gel Electrophoresis

To perform DNA gel electrophoresis 50 µl agorose gel containing 5 µl of SafeView were cast in the DNA gel rig. DNA samples were loaded into each of the wells of the 0.8-2% DNA agarose gel (depending on whether separation of larger (0.8% ~2-10 kb) or smaller (2% ~50-250 bp) DNA fragments). Depending on fragment size a 100 bp or 1 kb DNA ladder was loaded along with the samples. Samples were then typically run at 120 V for 30 minutes. Samples were visualized using a Gene Genius Bio-imaging System.

2.2.1.7 Site Directed Mutagenesis

Primers for site directed mutagenesis were designed using the QuickChange™ Stratagene program (http://www.genomics.agilent.com/primerDesignProgram.jsp). PCR using Pfu Taq polymerase, plasmid containing target genes and site directed mutagenesis primers was then run using the following conditions: 95°C, 1min; 56°C, 1.30 min; 68°C for 1 min per kb of plasmid to be amplified; Go back to Step 2, step 2 to 3 was then repeated for 20 cycles; 72°C, 5min and hold reaction at 4°C. PCR product was purified using the Qiagen QIAquick PCR Purification Kit and 1µg of the purified PCR product was then digested for 1 hour at 37^{o}C. The sample was then incubated at 62^{o}C for 20 minutes in order to inactivate the digest. The digest was transformed using as the ligation reaction transformation protocol. Colonies were screened for plasmid and sequences by GATC to confirm successful site directed mutagenesis. The primer pairs used to generate the SDM of the T2P 2.6 reporter can be found in table 2.II.

Mutation	Primer	Sequence
Mutation of E2F Binding Site A on the T2P 2.6 Reporter	Mut. A F	5'-acccggtattgaatgtgttctagtactaaatactgagatgccgggg-3'
	Mut. A R	5'-ccccggcatctcagtatttagtactagaacacattcaataccgggt-3'
Mutation of E2F Binding Site B on the T2P 2.6 Reporter	Mut. B F	5'-tgctggcagctcctgcaggacctttttttagctcctgctgctggtaccccc-3'
	Mut. B R	5'-gggggtaccagcagcaggagctaaaaaaaggtcctgcaggagctgccagca-3'
Mutation of E2F Binding Site C on the T2P 2.6 Reporter	Mut. C F	5'-cgggctctgccttgggctttgaaaaaatacctgtgcctgccctactcc-3'
	Mut. C R	5'-ggagtagggcaggcacaggtatttttttcaaagcccaaggcagagcccg-3'

Table 2.II: Primer pairs for construction of various T2P 2.6 mutants.

2.2.1.8 Cloning

The T2P 2.6 reporter was digested using the restriction enzyme Kpn. This resulted in the formation of a linarized T2P 2.6 reporter containing only the first 800 bp of the Trib2 promoter region. This DNA fragment was gel purified using the Qiagen QIAquick Gel Extraction Kit. The plasmid was then relegated together to form the T2P 800 by incubation of 100 ng of linarized plasmid with 1 µl of DNA ligase overnight at 16^{o}C. The sample was transformed using as the ligation reaction transformation protocol. Colonies were screened for plasmid and sequenced by GATC to confirm successful cloning of the T2P 800.

The T2P 927 the T2P 2.6 reporter was again digested with Kpn1. A fragment of DNA consisting of 127 bp of the Trib2 promoter region upstream of the region found in the T2P 800 was generated. This fragment was gel purified using the Qiagen QIAquick Gel Extraction Kit. A ration of 1:3 of this purified fragment to T2P 800 totalling 150 ng was prepared and incubated with 1 µl of DNA ligase overnight at 16^{o}C. The sample was transformed using as the ligation reaction transformation protocol. Colonies were screened for plasmid and sequenced by GATC to confirm successful cloning of the T2P 927.

In order to generate the T2P 963 reporter the reporter T2P Mutant (Mut.) C was amplified using Roche Fast Start High Fidelity Taq using the primers in table 2.III. The reaction was set up with 4 µl of the 5X Buffer, 0.4 µl of 10 mM dNTPs, 1 µl of 10 µM Forward Primer, 1 µl of 10 µM Reverse Primer, 1 µl of Template DNA (about 50 ng) and 0.2 µl of DNA Polymerase in a PCR eppindorf. The PCR eppindorf was then placed in a thermocyler machine and the PCR was performed under the following conditions; Initial Denaturation Step at 98°C for 30 seconds, followed by 30 cycles of 98°C for 10 seconds, 67oC for 10 seconds and 72°C for 30 seconds. The Final Extension Step was then performed at 72°C for 5-10 minutes.

Mutation	Primer	Sequence
T2P 963	T2P FP XhoI	5'-ctcgagttccacattccttccctctg-3'
	T2P RP XhoI	5'-gagctcgtgatgggggtagacctgtg-3'

Table 2.III: Primer pairs used in the cloning of T2P 963.

The amplified PCR product was gel purified by running the PCR sample on a gel and cutting out and extracting the amplified PCR product from the gel using a GenElute Gel Extraction Kit (Sigma). The ligation reaction was then set up in an eppindorf containing 1 µl (~0.02 pmol) of the **A**ccep**T**or™ Vector (50 ng/µl), 4 µl (between 0.1 and 0.2 pmol) of the PCR product and 5.0 µl Clonables™ 2X Ligation Premix. The ligation reaction was then incubated at 16°C for 2 hours. The ligation mixture was then spread on LB agar plates containing ampicilian and incubated overnight at 37^{o}C. A number of colonies were then selected and incubated overnight at 37^{o}C in LB broth with ampicilian. DNA was isolated from the colonies and screened for successful clones by digestion with XhoI. Once successful clone was identified this clone was digested with XhoI as was the pGL3 Basic vector. A ratio of 1:3 of pGL3 Basic to PCR fragment totalling 150 ng of DNA was prepared and

incubated with 1µl of DNA ligase overnight at 16°C. The sample was then transformed. Successful colonies were screened for plasmid and sequenced by GATC to confirm successful cloning of the T2P 963.

2.2.1.9 cDNA Preparation for Real-Time PCR

mRNA was isolated from pelleted cell samples using the Qiagen mRNeasy mini kit including the DNase digest step in the kit in order to remove all genomic DNA from the sample. 200ng of mRNA from each sample was then added along with 1µl of Random Hexamers (50ng/µl) and 1µl of 10mM dNTPs to a PCR test tube. The PCR test tubes were then run under the following conditions: 65°C for 5 minutes; 4°C for 5 minutes; 25°C for 15 minutes, 42°C for 50 minutes, 70°C for 15 minutes; 4°C forever on a PCR cycler. During the 4°C incubation the following buffers were added to each tube: 5X RT buffer (4µl), 25mM $MgCl_2$ (4µl), 0.1M Dithiothreitol (DTT) (2µl) and 1µl of RNase out. 2 minutes into the 25°C incubation step 1µl of SSIII reverse transcriptase (50U) was added to each PCR tube. Sample volume was brought up to 45µl by addition of ddH_2O and 2µl of each cDNA prep was then be used for Real-Time PCR analyses.

2.2.1.10 Real-Time PCR

10µl of 2xKAPA SYBR Master Mix or SensiMix SYBR No-ROX (Bioline), 0.5µl Forward Primer (100µmolar), 0.5µl Reverse Primer (100µmolar), 7µl of ddH2O and 2µl of the cDNA Sample were plated in duplicate on a 96 well PCR plate. Real-Time PCR was then performed under the following conditions on the Opticon Real-Time machine: 15 minutes at 95°C; 15 seconds at 95°C; 60 seconds at 60°C followed by plate read; step 2 to 3 the was then repeated 39 more times; 5 minutes at 72°C; 1 minutes at 95°C; melting curve 65 – 95°C, read every 0.5°C, hold 15 seconds

between reads; infinity at 20°C. Target gene levels were then normalised to 18S (control) using the $2^{-\Delta\Delta CT}$ method. Table 2.IV contains the list of primer pairs used in the real-time analyses.

Target Gene	Name	Target Species	Sequence
18S	18S F	Mouse and Human	5'-GCGATGCGGCGGCGTTATTC-3'
	18S R	Mouse and Human	5'-GCCGGGTGAGGTTTCCCGTG-3'
Trib1	Trib1 F	Mouse	5'-TCCTATGTGCGGAGCCGAAA-3'
	Trib1 R	Mouse	5'-GACGGCGGAAACAATCTGCTTGAA-3'
Trib2	Trib2 F	Murine	5'-AGCCCGACTGTTCTACCAGA-3'
	Trib2 R	Murine	5'-AGCGTCTTCCAAACTCTCCA-3'
Trib2	Trib2 F	Human	5'-CAAGCTGCGGAAATTCATCT-3'
	Trib2 R	Human	5'-GTAGCTGCCACTGGTGTTCA-3'

Table 2.IV: Details of primers used in real-time PCR analyses.

TRIB1 Qiagen QuantiTect primers for the human TRIB1 gene were used to analyses human TRIB1 gene expression.

2.2.2 Cell Biology

2.2.2.1 Transfection

Transfections were carried out using Turbofect transfection reagent (Thermo Scientific Pierce) (details of the transfection protocols used for Turbofect is detailed in the Luciferase assay section (2.2.2.2)) or Attractene Transfection reagent (Qiagen).

Attractene Transfection reagent (Qiagen) was used to transfect the wild-type and E2F1 knock-out MEF cells. The MEF cells were plated at 1.6 x 10^6 per 100 mm 24

dish 24 hours before transfection. 4 μg of DNA was incubated with 15 μl of attartene reagent with DMEM in a solution of total volume of 300 μl. The sample was incubated for 10 to 15 minutes at room temperature. During this time the media on the cells was changed for fresh media. Post incubation the transfection media was added to the plate dropwise and the plate swirled to distribute the transfection mixture. The cells were then incubated for 24 hours before being trypsonised and collected and analysed for protein.

For RNA analysis the cells were also transfected with attractene, this time in a 6-well dish format. 0.4 x 10^6 cells were seeded per well 24 hours pre-transfection. The protocol is the same as for the 100 mm dish. However 2 μg of DNA and 12.5 μl of attractene reagent were incubated in a total volume of 100 μl DMEM before being added to each well.

2.2.2.2 Luciferase Assay

3T3 cells were transfected in duplicate using TurboFect Transfection Reagent in a 24 well plate. 20,000 3T3 cells were plated per well. Each well was transfected with 100 ng of reporter (e.g. T2P 2.6 or pGL3Basic (control reporter)), 400 ng of expression vector (e.g. E2F1 plasmid or empty plasmid pcDNA3 (control)) and 10 ng of pRL-TK (internal control) as follows. The 500 ng DNA mixture was made up to a total volume of 5 μl using ddH_2O. 45 μl of DMEM was then added to this DNA mixture. 1 μl of Turbofect reagent was then added to the sample mixture which was incubated for between 15 and 20 minutes at room temperature. Post incubation the transfection all 50 μl of the DNA Turbofect mixture was then added to the well dropwise. The cells were than incubated overnight at 37^oC in a cell incubator. 24 hours post-transfection cells were lysed using the passive lysis buffer at room

temperature. Firefly luciferase activity from the relevant promoter construct (pGL3-Basic, T2P 500, T2P 2.6, etc.) and Renilla luciferase activity from the internal control plasmid pRL-TK were determined using a dual-luciferase reporter assay system as previously described (Dyer et al., 2000). For all samples the firefly luciferase activity was divided by the activity of the Renilla luciferase in order to control for transfection efficiency.

2.2.2.3 Chip Assay

The Chip assay was performed using the Active Motif ChIP-IT Express Chromatin Immunoprecipitation Kit as follows. K562 cells were grown in 10 ml flasks for 2 days; until cells were confluent. 270 µl of 37% formaldehyde was then added to the confluent K562 cells which were then incubated for 10 minutes on a rocker at room temperature in order to cross-link their DNA. Glycine (1.25M) was added to the cells after the 10 minute incubation and the cells were further incubated for 5 minutes at room temperature on a shaker in order to stop the cell fixation (DNA cross-linking) by the formaldehyde. The cells were then pelleted by centrifugation at 1500 rpm in an Eppindorf 5415 R Centrifuge for 5 minutes at 4ºC, washed in 2 ml of ice cold Phosphate Buffered Saline (PBS) and 10 µl 100 mM of phenylmethylsulfonyl fluoride (PMSF) and finally pelleted by centrifugation at 2500 rpm in an Eppindorf 5415 R Centrifuge for 10 min at 4ºC. At this point the cells were stored at -80^{o}C if thier chromatin was not immediately isolated.

If necessary the pellet was thawed and then resuspend in 1 ml of ice cold Lysis buffer supplemented with 5 µl of Protease Inhibitor Cocktail (PIC) and 5 µl PMSF. The sample was then incubated on ice for 30 minutes. Post incubation the samples was transfered to an ice cold dounce homogenizer and gently dounced on ice with 10

strokes to aid in nuclei release. The sample was then transfered to an eppindorf and centrifuged at 5000 rpm in an Eppindorf 5415 R Centrifuge for 10 minutes at 4ºC in order to pellet nuclei. The supernatant was then carefully removed and discarded. The nuclei pellet was then resuspended in 350 µl of Shearing buffer which was supplemented with 1.75 µl of PIC. This sample was then placed on ice.

Using a sonicator the DNA of the resuspended nuclei was sheared using optimised sonication conditions for K562 cells of 10 pulses each lasting 20 seconds in length with 30 seconds of rest for the sample on ice between each pulse. This sheared chromatin was then centrifuged at 15,000 rpm in an Eppindorf 5415 R Centrifuge for 10 min at 4ºC. Finally the sheared chromatin was transferred to a fresh eppindorf and either used straight away or stored at -80ºC.

If necessary the chromatin was thawed and 10 µl of the chromatin was transferred into a separate eppindorf labelled INPUT. Next the Chromatin Immunopricipitation reaction was set up as follows: 25 µl of magnetic Protein G Beads, 20 µl of ChIP Buffer 1, 100 µl of the K562 chromatin, 2 µl of PIC and 2 µg of the relevant antibody or Rabbit IgG control were all placed in a silicone coated eppindorf. The antibodies listed in Table 2.V were used for the Chip analyses.

Antibody	Manufactorer	Code	Concentration
E2F1 (c-20)	Santa cruz	sc-193X	2µg
E2F2 (c-20)	Santa cruz	sc-633X	2µg
E2F3 (N-20)	Santa cruz	sc-879X	2µg
E2F4 (c-20)	Santa cruz	sc-866X	2µg
E2F5 (c-20)	Santa cruz	sc-1083x	2µg

Table 2.V: Antibodies used for the Chromatin Immunoprecipitation experiments.

The samples were mixed and incubated on an end-to-end rotator at 4ºC overnight. The samples were then spun down and placed on a magnetic stand allowing the beads to gather at one side of the tube. The supernatant was then carefully removed and discarded. The magnetic beads were then washed once with 800 µl Chip Buffer 1 and then twice with 800 µl of Chip Buffer 2. As much supernatant as possible was removed after the final wash. The beads were then resuspend with 50 µl of Elution Buffer AM2 and incubated at room temperature on an end-to-end rotator for 15 minutes. The samples were quickly spun and then 50 µl of Reverse cross-linking Buffer was added to elute the chromatin. The sample was immediately placed on the magnetic stand, allowing the beads to form a pellet. The supernatant was transferred to a fresh eppindorf. At this point the IPNUT sample was added to 88 µl ChIP buffer 2 and 2 µl 5 M NaCl, so that its final volume is 100 µl. All samples were then incubated at 65º C for 4 hours. Once again the samples were spun down and 2 µl of Proteinase K were added to them. The samples were then mixed and incubated for 1 hour at 37ºC (during this time the Proteinase K Stop Solution was incubated at room temperature for up to 1 hour). The samples were then returned to room temperature and 2 µl of Proteinase K Stop Solution was added to them. The DNA was now ready to be used for PCR analysis. Real-time PCR analyses of the DNA pull down using target genes for the TRIB2 promoter and a control region using the standard curve method as per section 2.2.1.10. Primers used for Real-Time PCR are detailed in table 2.VI.

Primers	Sequence
Trib2 Promoter F	5'-GGG GAG ACG GGG TGA TTG CA-3'
Trib2 Promoter R	5'-CGG GCT GGG CGC AGG TA-3'
Control (-5kb upstream of Trib2 transcriptional start sight) F	5'-AGG TGT GCA CCT CTT CCC TGA-3'
Control (-5kb upstream of Trib2 transcriptional start sight) R	5'-TGG GAA ACC CAA TCA GGA TAC ATC C-3'

Table 2.VI: Real-time PCR primers used for assessment of DNA pull-down in the Chromatin Immunoprecipitation experiments.

To asses pull-down the beads were boiled after immunopercipitation in 20 µl of 2 x loading buffer. 10 µl of this sample for both antibody and IgG control were run on a western blot along with the input chromatin as per procedure in section 2.2.2.4.

2.2.2.4 Western Blot

For the MEFs, cells were lysed in Radioimmunoprecipitation Assay (RIPA) lyses buffer and equivalent concentrations of lysates were denatured by addition of sodium dodecyl sulfate (SDS) loading buffer and boiling for 5 minutes on a heating block. Similarly, the magnetic beads for immunopercipitation were boiled in SDS loading buffer for 5 minutes on a heating block. Samples were resolved on 12 % (w/v) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to nitrocellulose membrane (Sigma). Following transfer, membranes were blocked for 1 hour at room temperature in 5 % (w/v) Marvel milk/PBS-Tween 0.1 % (v/v). Primary antibodies were diluted as outlined below in 5 % (w/v) Marvel milk/PBS-Tween 0.1 % (v/v) or 2 % (w/v) Marvel milk/PBS-Tween 0.1 % (v/v) for the Trib2 antibody and incubated on the shaking membranes at 4°C overnight or for 1 hour at room temperature as indicated in table 2.VII.

Antibody Name	Manufactorer	Company Code	Working Dilution	Incubation Conditions
Trib2(b-06)	Santa cruz	Sc-100878	1/100	4°C Overnight
E2F1 (c-20)	Santa cruz	sc-193	1/1000	1 hour at room temperature
Actin	Sigma	A5541	1/10000	1 hour at room temperature
E2F1 (c-20)	Santa cruz	sc-193X	1/2000	4°C Overnight
E2F2 (c-20)	Santa cruz	sc-633X	1/2000	4°C Overnight
E2F3 (N-20)	Santa cruz	sc-879X	1/2000	4°C Overnight
E2F4 (c-20)	Santa cruz	sc-866X	1/2000	4°C Overnight
E2F5	Santa cruz		1/2000	4°C Overnight

Table 2.VII: Antibodies used in western blot analysis.

Membranes were washed three times in PBS-Tween for 5 minutes each and incubated in diluted secondary antibody for 1 hour at room temperature using 1:1000 dilution of HRP conjugated secondary antibodies in 5 % (w/v) Marvel milk/PBS-Tween. Following washing, proteins were detected by autoradiography with the ECL reagent for HRP conjugated secondary antibodies. Densitometric analysis was carried out using Image J software (version 1.46) as per the developer's protocol (Schneider et al., 2012).

2.2.2.5 K562-C/EBPα-ER Induction

To induce expression of C/EBPα in the K562-C/EBPα-ER cell lines cells (1×10^6) were induced by addition of 5 μM β-estradiol (Sigma-Aldrich) dissolved in ethanol or control (ethanol only) to the cell culture media. Cells were harvested at the various time points post stimulation for real-time analyses of the genes of interest.

2.2.2.6 Serum Stimulation

Cells were cultured in serum free media for 24 hours. 10% FBS was then added to the media and cells were harvested at the various time points post stimulation for real-time analyses of the genes of interest.

2.2.3 Bioinformatics

2.2.3.1 Data Sources and Expression Profiling

Two major microarray datasets are analysed in this thesis, the MILE (Microarray Innovations in Leukaemia) Study dataset (GSE13204) (Haferlach et al., 2010) and a microarray dataset of gene expression of the cells of haematopoiesis (GSE24759) (Novershtern et al., 2011). Both datasets are publically available and can be downloaded from the Gene Expression Omnibus (GEO).

In order to generate expression profiles for the genes of interests the datasets were first collapsed to maximum probe expression followed by row normalization of the datasets. This was done to give a single expression profile for each gene derived from the multiple probes available for each gene in the geneset. For each collapsed gene probe of interest the expression levels of the gene and values for each leukaemia subtype and control group, in the case of the MILE study, or of the normal cell of haematopoiesis (Novershtern et al., 2011) were plotted on a box plot with maximum and minimum whiskers using GraphPad

2.2.3.2 Generation of gene lists and heat maps

To determine the nearest-neighbours both in the MILE dataset and for each of the subtypes of leukaemia and in the normal cells of haematopoiesis the unclollapsed

datasets were analysed. First each sample was separated based on whether the expression of the genes of interest were below the median expression or above the median expression for all of the probe sets available for each gene of interest in the dataset. Next a one-way analysis of variance (ANOVA) analysis was performed to determine the genes with significantly differential expression between the two groups (below versus above the median for all probe sets). Unsupervised hierarchical clustering of the top 1000 differentially expressed genes that were determined by ANOVA analysis was then performed using PARTEK GENOMICS SUITE (Version 6.6).

2.2.3.3 Comparative Marker Selection

Genesets of the top differentially expressed genes in the Wouters (GSE14468) (Wouters et al., 2009) and Valk (GSE1159) (Valk et al., 2004) datasets for Acute Promyelocytic Leukaemia (APL) versus AML and in the Valk dataset for APL versus the non-leukaemic samples and CEBPA mutated versus wildtype CEBPA were generated using the comparative marker selection tool (version 9) hosted on the genepattern platform (Reich et al., 2006). The Microarray dataset was uploaded into gene pattern for either the Valk or Wouters dataset as a .gct file along with a class file (.cls) which indicated which samples were APL or AML and which samples had wildtype or mutated CEBPA. The analysis was then run with 1000 permutations for the AML versus the APL or wildtype versus mutated CEBPA with a random seed number. The top differentially expressed genes were selected based on p-value.

2.2.3.4 GSEA

Gene set enrichment analysis (GSEA) with 1000 permutations was carried out using the GSEA program suite (version 2.07) available from the Broad Institute (http://www.broadinstitute.org/gsea/index.jsp) (Subramanian et al., 2005). First the microarray dataset was loaded as a .gct file into the GSEA program suite along with a continuous label phenotype file (.cls) containing the information for the continuous label (gene expression profile of gene of interest in the microarray). A geneset file was also loaded (.gmx or .gmt) and these files were sourced either from the Molecular Signalling Database (MSigDB) (v3.0), were comprised of sets of genes as determined using comparative marker selection analysis or were comprised of the 80 modules of strongly coexpressed genes identified by Novershtern et al (2011) in the haematopoietic cell lineages. In the GSEA program suite the microarray file was first collapsed to maximum probe expression before running the analysis. The analysis was then run using the collapsed microarray dataset and the phenotype and gene sets of interest. The microarray genes were ranked during the continuous phenotype analysis using the Pearson correlation matrix with continuous signature labels for the gene of interest and run against the genesets. Details on how to create the relevant files from the microarray datasets and how to run the analysis can be found here http://www.broadinstitute.org/gsea/doc/GSEAUserGuide.pdf.

2.2.3.5 SSC Mapping

In order to perform connectivity mapping (SSC Mapping) the top 50 nearest-neighbours for the genes of interest for each of the subtypes of leukaemia in the MILE dataset and the cells of haematopoiesis from section 2.2.3.2 were utilised to create a gene signature for high expression of the gene of interest. These nearest-

neighbour genes were separated based on whether the genes were negative or positive neighbours and given a value of 1 or -1 depending on whether they were positive or negative gene neighbours. The gene neighbour files were then uploaded as .grp files into the online SCC mapping program and the analysis was run. Any gene probe in the gene neighbour file that was not present in the feature set was automatically removed from the analysis. The analyses was then carried using the connectivity mapping program (Broad Institute) which along with a user manual for the program is available online at http://www.broadinstitute.org/scientific-community/science/projects/connectivity-map/connectivity-map (Lamb et al., 2006).

2.2.3.6 Transcription Factor Binding Sites Analysis

The promoter region for TRIB2 was found in the USCS genome browser (http://genome-euro.ucsc.edu/cgi-bin/hgGateway?redirect=auto&source=genome.ucsc.edu). Analysis of the promoter region of TRIB2 for potential transcription factors binding sites was performed using the online the online tool TESS (Transcription Element Search System). Available at http://www.cbil.upenn.edu/tess (Schug and Overton, 1997).

2.2.3.7 Correlation Analysis

Correlation analysis was carried out in GraphPad Prism 5 using gene expression data derived from the Valk (GSE1159) (Valk et al., 2004) and MILE (GSE13204) (Haferlach et al., 2010) datasets. Expressions of the patient samples were plotted and the line of best fit calculated by linear regression using GraphPad Prism 5.

Chapter 3

Profiling of TRIB1, TRIB2 and TRIB3 Expression in Leukaemia and in the Normal Cells of Haematopoiesis

This work was published in part in British Journal of Haematology 158(5) 626-34; September 2012 entitled "Elevated TRIB2 with NOTCH1 activation in paediatric/adult T-ALL" Hannon MM, Lohan F, Erbilgin Y et al. (Appendix A).

Introduction

The Tribble family of proteins consists of three members, TRIB1, TRIB2 and TRIB3. These Tribble proteins are psudokinases (Hegedus et al., 2007) that, to date, have been shown to be involved in the mediation of a vast array of cellular and physiological processes including proliferation, differentiation, haematopoiesis, regulation of cell migration, adipogenesis, apoptosis and protein degradation (Yokoyama and Nakamura, 2011; Liang et al., 2013). There is growing and substantive evidence that the Tribble genes are oncogenes involved in myelopoiesis and tumourigenesis. The Tribble genes have also been linked to non-neoplastic disorders such as cardiovascular disease and metabolic disorders (Yokoyama and Nakamura, 2011; Angyal and Kiss-Toth, 2012).

Expression of each member of the Tribble family has been widely linked to cancer. For example TRIB1 expression has been shown to correlate with prognosis in ovarian cancer (Puiffe et al., 2007), TRIB2 expression has been linked to lung cancer (Grandinetti et al., 2011; Zhang et al., 2012) and melanoma (Zanella et al., 2010) and TRIB3 expression is associated with prognosis in both colorectal (Miyoshi et al., 2009) and breast cancer (Wennemers et al., 2011a, 2011b). But it was as leukaemia causing genes that the Tribbles were first identified. Retroviral expression of Trib1 or Trib2, but not Trib3, in murine bone marrow progenitors resulted in the development of AML (Keeshan et al., 2006; Jin et al., 2007; Dedhia et al., 2010). High levels of TRIB1 and TRIB2 expression have both been connected to human haematopoietic malignancies. Enhanced TRIB2 expression was found to identify with a subset of normal karyotype AML with silenced CEBPA expression and activated NOTCH1 expression. TRIB2 was identified as a NOTCH1 target and this leukaemia phenotype also possessed aberrant expression of T-cell genes (Keeshan et

al., 2006; Wouters et al., 2007). Increased TRIB2 expression is associated with poor prognosis in CLL (Johansson et al., 2010). TRIB1 expression has been found to be elevated in AML and in myelodysplastic syndrome patient samples (Storlazzi et al., 2004; Rücker et al., 2006; Röthlisberger et al., 2007).

The Microarray Innovations in Leukaemia (MILE) study was a collaborative international program whose aim was to assess the clinical utility and accuracy of microarray gene expression profiles. Thousands of leukaemic and myelodysplastic syndromes (MDS) patient samples were collected by 11 different labs located in Europe, the United States of America and Singapore. The samples were classified into MDS or into 16 acute and chronic leukaemia subclasses. Non-leukaemic and normal bone marrow samples were also collected for use as a control group in the analysis (Haferlach et al., 2010).

In 2011 Novershtern et al. published a microarray dataset composed of the gene expression profiles of 38 purified human cell populations (or cellular compartments) of the haematopoietic system. This dataset included the HSCs, a number of different progenitor cells and twelve mature haematopoietic cell types. Both the MILE study dataset and the microarray datasets of the cellular compartments of haematopoiesis are freely available online and can be utilized for further analysis of the gene expression profiles of both human haematopoiesis and the leukaemic subtypes.

Here the expression of TRIB1, TRIB2 and TRIB3 was profiled in the leukaemic subtypes of the MILE study (Haferlach et al., 2010) and in the cellular compartments of haematopoiesis (Novershtern et al., 2011). While TRIB1 expression was not found to be significantly increased in any of the leukaemic subsets compared to expression in the control group cells, TRIB2 expression was found to be significantly higher in the leukaemic T-ALL and ALL with t(1;19) samples. In the

cellular compartments of haematopoiesis TRIB1 expression was highest in the myeloid compartment and the TRIB1 signature in leukaemia is enriched with genes closely associated with myeloid cell expression. In contrast TRIB2 expression was highest in the lymphoid compartment and the TRIB2 signature in leukaemia is enriched with genes most closely related to lymphoid cell expression. Although TRIB3 over-expression in the murine bone-marrow progenitor cells does not lead to the development of AML (Dedhia et al., 2010) significant increases in TRIB3 expression levels were observed in the AML with complex and aberrant karyotype, CML and the MDS patient samples compared to the control group samples.

The Tribble genes have already been identified as mediators of adipocyte differentiation (Naiki et al., 2007). Mounting evidence suggests that these genes may also play a role in the mediation of haematopoiesis. Trib1-deficient mice show lack tissue-resident M2-like macrophages (associated with responses to anti-inflammatory reactions and tumour progression) and eosinophils due to aberrant C/EBPα expression (Satoh et al., 2013). This indicates that TRIB1 expression is a key factor in myelopoesis. Analyses of Trib2, and Trib3, deficient mice revealed no defects in either the myeloid cells or the lymphoid cells in the spleen (Satoh et al., 2013). However both Trib1 and Trib2 were found to mediate the degradation of the C/EBPα protein via its COP1 domain in leukaemic cells and non-leukaemic cells of haematopoiesis (Keeshan et al., 2010; Yokoyama et al., 2010; Satoh et al., 2013). C/EBPα is a key myeloid differentiation factor involved in granulopoiesis and monopoiesis. Depletion of C/EBPα protein levels or mutation of the C/EBPα gene in the myeloid progenitor cell has been identified as a contributing factor to myeloid transformation. Aberrant C/EBPα expression results in a block in myeloid differentiation and a promotion of myeloid progenitor cell cycle progression (Ramji

and Foka, 2002; Paz-Priel and Friedman, 2011). TRIB2 has also been identified as a major hub gene (that is a highly interconnected gene in a regulatory network) in human B cells indicating that is plays an important regulatory role in those cells (Basso et al., 2005).

Results

3.1 Expression levels of TRIB1, TRIB2 and TRIB3 in the subtypes of leukaemia and the cells of normal haematopoiesis

Analyses of TRIB1, TRIB2 and TRIB3 expression in the subtypes of leukaemia found in the MILE study and in the cells of normal haematopoiesis revealed distinct differences in TRIB1, TRIB2 and TRIB3 expression across the two datasets (figure 3.1, 3.2 and 3.3). Both datasets were downloaded from GEO and collapsed to maximum probe expression for each gene analysed in the dataset using Gene Pattern. Gene expression in the dataset was then median-centred in order to bring all centres in the dataarray together in a pre-processing step, again using Gene Pattern, before expression of TRIB1, 2 and 3 was entered into GraphPad Prism 5 where it was plotted and statistically analysed.

Analyses of TRIB1 expression in the leukaemia subtypes (figure 3.1) found it to be higher than that of its expression in the control samples, which includes healthy bone marrow samples as well as samples from non-leukaemia conditions such as megaloblastic anaemia, haemolysis, iron deficiency and idiopathic thrombocytopenic purpura. In fact TRIB1 expression was found to be significantly lower in all of the ALL subtypes excluding mature B-ALL t(8;14). TRIB1 expression was also found to be significantly lower in the AML t(15;17), in the AML with normal karyotype and other abnormalities and in the CLL patient samples when compared to TRIB1 expression in the control group. Since TRIB1 expression has been reported to be elevated in AML and myelodysplastic syndrome (Storlazzi et al., 2004; Rücker et al., 2006; Röthlisberger et al., 2007) elevated TRIB1 expression would be expected in at

least a subset of the AML patient samples. This was found not to be case (Figures 3.1). Though, overall TRIB1 expression is higher in the AML subsets compared to the ALL subsets (figure 3.1).

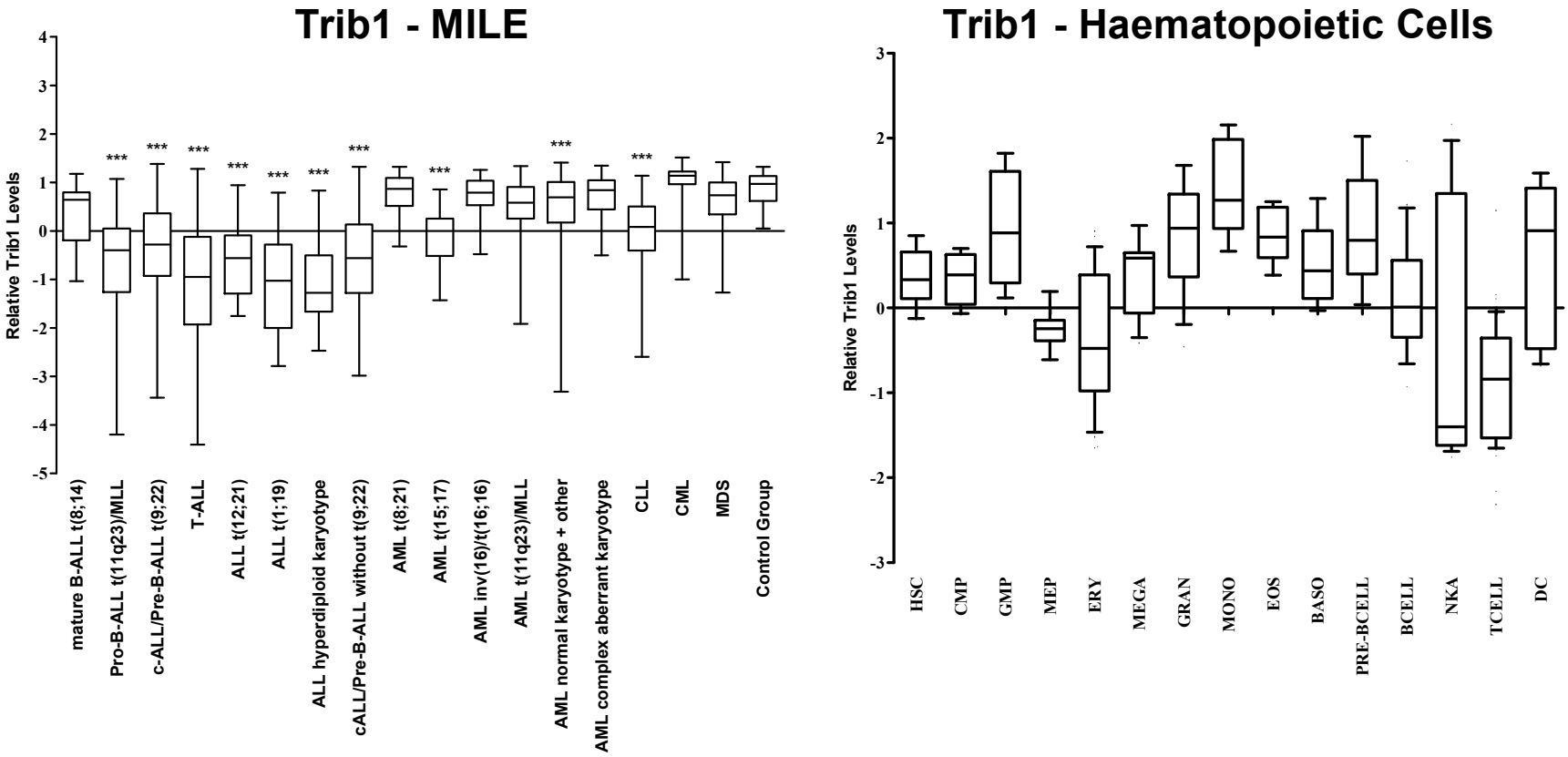

Figure 3.1: Expression profiles of TRIB1 expression in each subset of leukaemia and in the control group and in the cells of haematopoiesis. Mature B-ALL t(8;14), N = 14; Pro-B-ALL t(11q23)/MLL, N = 70; c-ALL/Pre-B-ALL t(9;22), N = 122; T-ALL, N = 174; ALL t(12;21), N = 58; ALL t(1;19), N = 36; ALL hyperdiploid karyotype, N = 40; cALL/Pre-B-ALL without t(9;22), N = 237; AML t(8;21), N = 40; AML t(15;17), N = 37; AML inv(16)/t(16;16), N = 28; AML t(11q23)/MLL, N = 38; AML normal karyotype + other, N = 351; AML complex aberrant karyotype, N = 48; CLL, N = 448; CML, N = 76; MDS, N = 206; Control Group, N = 74; HSC = haematopoietic stem cell, N = 14; CMP = common myeloid progenitor, N = 4; GMP = granulocyte monocyte progenitor, N = 4; MEP = megakaryocyte/erythroid progenitor, N = 9; ERY = erythroid cells, N = 33; MEGA = megakaryocytes, N = 12; GRAN = neutrophils, N = 13; MONO = monocytes, N = 9; EOS =

eosinophils, N = 5; BASO = basophils, N = 6; PREBCELL = Pre and Pro B Cells, N = 9; BCELL = B cells, N = 20; NKA = natural killer cells, N = 18; TCELL = T cells, N = 45; DC = dendritic cells, N = 10. Samples are plotted as a boxes and whiskers graph. The box extends from the 25th to 75th percentiles and the line in the boxes represents the median value of the samples. The whiskers extend from the maximum to the minimum sample value. Statistical analyses was carried out by performing a one-way ANOVA analyses followed by a Bonferroni's Multiple Comparison Test of non-leukaemic and healthy bone marrow versus each of the leukaemic subtypes using GraphPad Prism 5. A p-value below 0.05 indicated a significant difference in gene expression between the non-leukaemic and healthy bone marrow samples and the leukaemic subtypes, p-value below 0.05 is represented by *, below 0.01 by ** and below 0.001 by ***.

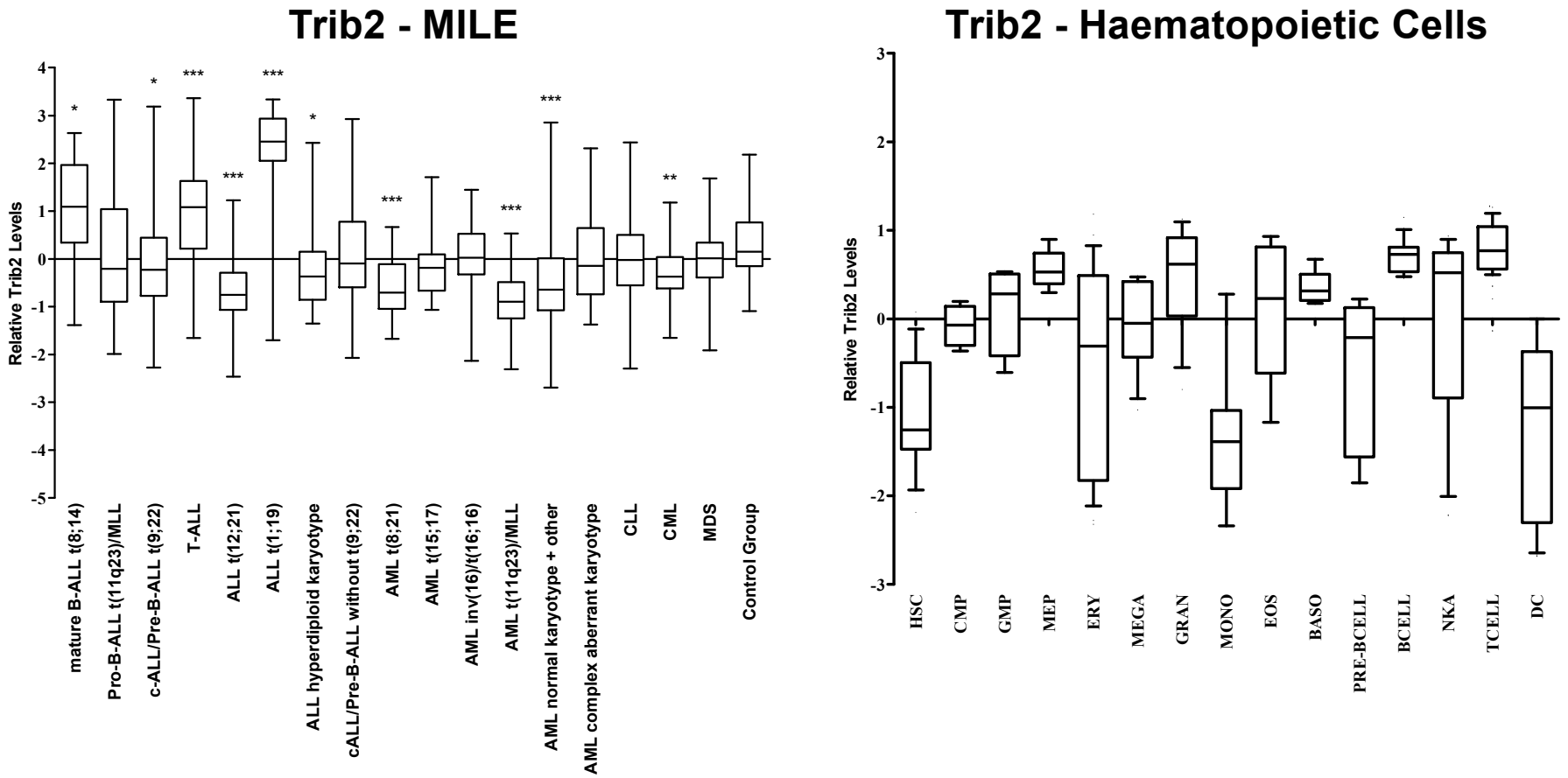

Figure 3.2: Expression profiles of TRIB2 expression in each subset of leukaemia and in the control group and in the cells of haematopoiesis. Mature B-ALL t(8;14), N = 14; Pro-B-ALL t(11q23)/MLL, N = 70; c-ALL/Pre-B-ALL t(9;22), N = 122; T-ALL, N = 174; ALL t(12;21), N = 58; ALL t(1;19), N = 36; ALL hyperdiploid karyotype, N = 40; cALL/Pre-B-ALL without t(9;22), N = 237; AML t(8;21), N = 40; AML

t(15;17), N = 37; AML inv(16)/t(16;16), N = 28; AML t(11q23)/MLL, N = 38; AML normal karyotype + other, N = 351; AML complex aberrant karyotype, N = 48; CLL, N = 448; CML, N = 76; MDS, N = 206; Control Group, N = 74; HSC = haematopoietic stem cell, N = 14; CMP = common myeloid progenitor, N = 4; GMP = granulocyte monocyte progenitor, N = 4; MEP = megakaryocyte/erythroid progenitor, N = 9; ERY = erythroid cells, N = 33; MEGA = megakaryocytes, N = 12; GRAN = neutrophils, N = 13; MONO = monocytes, N = 9; EOS = eosinophils, N = 5; BASO = basophils, N = 6; PREBCELL = Pre and Pro B Cells, N = 9; BCELL = B cells, N = 20; NKA = natural killer cells, N = 18; TCELL = T cells, N = 45; DC = dendritic cells, N = 10. Samples are plotted as a boxes and whiskers graph. The box extends from the 25th to 75th percentiles and the line in the boxes represents the median value of the samples. The whiskers extend from the maximum to the minimum sample value. Statistical analyses was carried out by performing a one-way ANOVA analyses followed by a Bonferroni's Multiple Comparison Test of non-leukaemic and healthy bone marrow versus each of the leukaemic subtypes using GraphPad Prism 5. A p-value below 0.05 indicated a significant difference in gene expression between the non-leukaemic and healthy bone marrow samples and the leukaemic subtypes, p-value below 0.05 is represented by *, below 0.01 by ** and below 0.001 by ***.

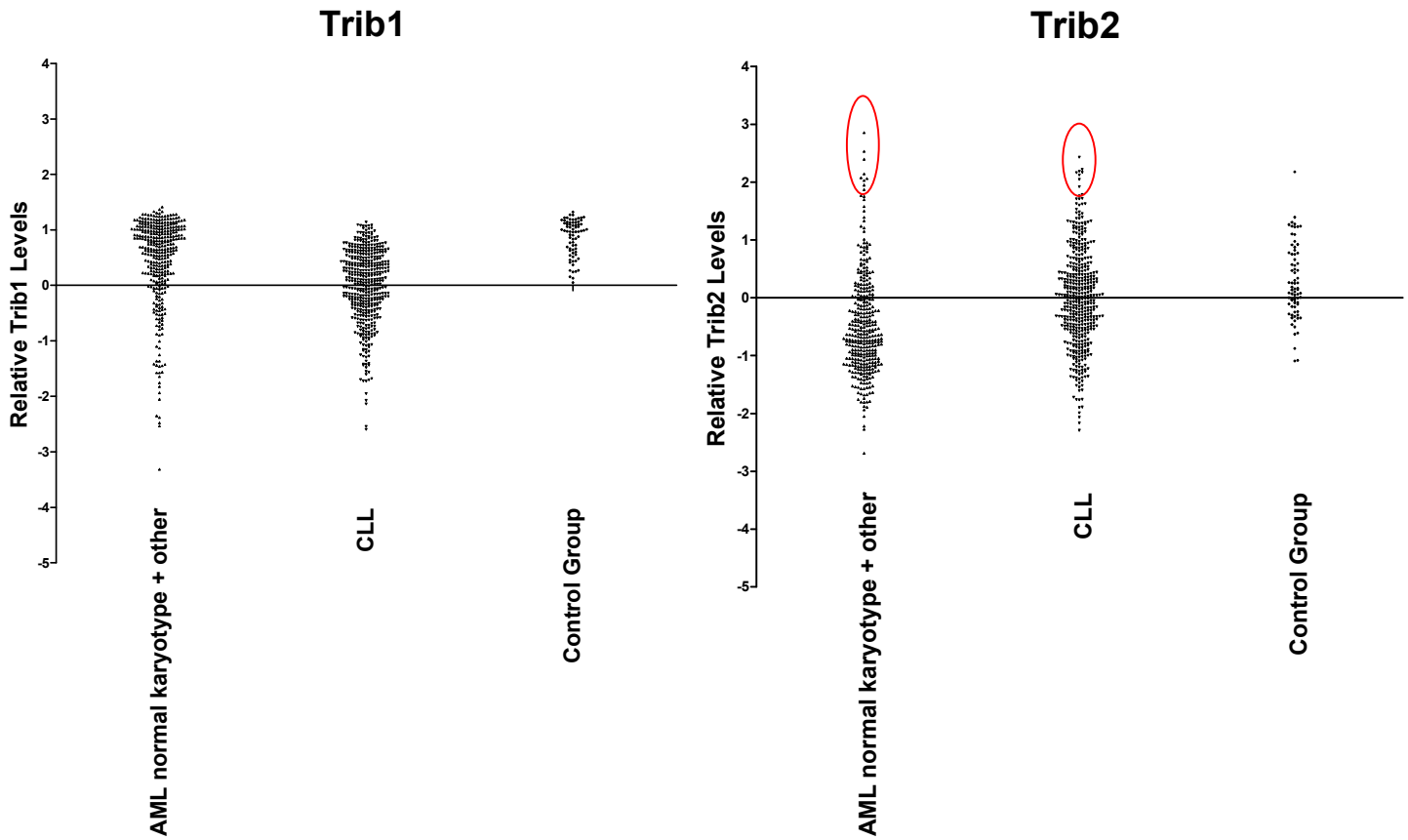

Figure 3.3: TRIB1 and TRIB2 expression in the AML with normal karyotype and other abnormalities, in the CLL and in the control group (healthy bone marrow samples as well as samples from non-leukaemia conditions such as megaloblastic anaemia, haemolysis, iron deficiency, or idiopathic thrombocytopenic purpura) patient samples. Samples are plotted as a vertical scatter plot. Patients in red circle for AML with normal karyotype, N = 14; Patients in red circle for CLL, N = 22. Subset of patients with increased TRIB2 expression compared to the control group samples are circled in red

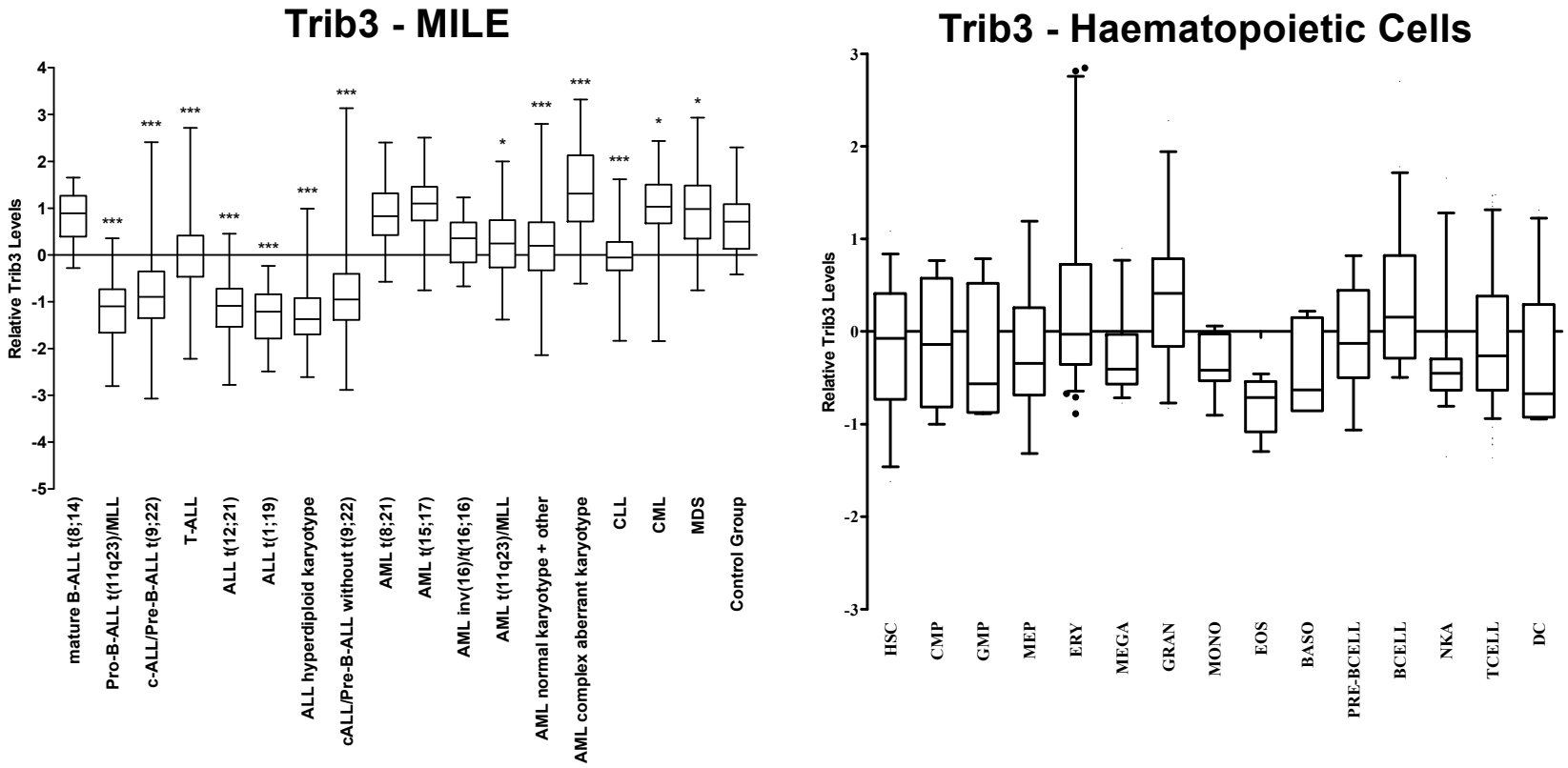

Figure 3.4: Expression profiles TRIB3 expression. in each subset of leukaemia and in the control group and in the normal cells of haematopoiesis. Mature B-ALL t(8;14), N = 14; Pro-B-ALL t(11q23)/MLL, N = 70; c-ALL/Pre-B-ALL t(9;22), N = 122; T-ALL, N = 174; ALL t(12;21), N = 58; ALL t(1;19), N = 36; ALL hyperdiploid karyotype, N = 40; cALL/Pre-B-ALL without t(9;22), N = 237; AML t(8;21), N = 40; AML t(15;17), N = 37; AML inv(16)/t(16;16), N = 28; AML t(11q23)/MLL, N = 38; AML normal karyotype + other, N = 351; AML complex aberrant karyotype, N = 48; CLL, N = 448; CML, N = 76; MDS, N = 206; Control Group, N = 74. HSC = haematopoietic stem cell, N = 14;

CMP = common myeloid progenitor, N = 4; GMP = granulocyte monocyte progenitor, N = 4; MEP = megakaryocyte/erythroid progenitor, N = 9; ERY = erythroid cells, N = 33; MEGA = megakaryocytes, N = 12; GRAN = neutrophils, N = 13; MONO = monocytes, N = 9; EOS = eosinophils, N = 5; BASO = basophils, N = 6; PREBCELL = Pre and Pro B Cells, N = 9; BCELL = B cells, N = 20; NKA = natural killer cells, N = 18; TCELL = T cells, N = 45; DC = dendritic cells, N = 10. Samples are plotted as a boxes and whiskers graph. The box extends from the 25th to 75th percentiles and the line in the boxes represents the median value of the samples. The whiskers extend from the maximum to the minimum sample value excluding the TRIB3 samples in haematopoiesis where the whiskers extend from the 90^{th} to the 10^{th} percentiles. Statistical analyses was carried out by performing a one-way ANOVA analyses followed by a Bonferroni's Multiple Comparison Test of non-leukaemic and healthy bone marrow versus each of the leukaemic subtypes using GraphPad Prism 5. A p-value below 0.05 indicated a significant difference in gene expression between the non-leukaemic and healthy bone marrow samples and the leukaemic subtypes, p-value below 0.05 is represented by *, below 0.01 by ** and below 0.001 by ***

When the same analysis was performed on TRIB2 its expression it was found to be significantly higher in the mature B-ALL t(8;14) samples, the T-ALL samples and the ALL t(1;19) patient samples when compared to TRIB2 expression in the control group (figure 3.1 (a)). In the c-ALL/Pre-B-ALL t(9;22), the ALL(12;21), the AML t(8;21), the AML t(11q23)/MLL, the AML with normal karyotype and other abnormalities and in the CML patient samples TRIB2 expression was found to be significantly lower than that of the control group (figure 3.2).

As previously stated elevated Trib2 expression causes murine AML and is highly expressed in a subset of AML patient samples with normal karyotypes (Keeshan et al., 2006). Direct comparison of TRIB2 expression in the AML patient samples with normal karyotype and the control group showed overall a decreased level of TRIB2 expression in the AML samples (figure 3.2). Plotting the AML patient samples with normal karyotype and the control group samples as a scatter plot revealed a subset of normal karyotype samples with TRIB2 expression elevated above those of the control group (figure 3.3). The same can be seen in the CLL patient samples, in which a small subset of patient samples was found to poses increased TRIB2 expression levels compared to the control group (figure 3.3). Data has been published implicating elevated TRIB2 expression in the disease progression of high risk patients with CLL (Johansson et al., 2010).

Unlike TRIB1, TRIB3 expression is found to be higher in the AML with complex and aberrant karyotype, CML and the MDS patient samples when compared to TRIB3 expression in the respective control group. Like TRIB1 however, TRIB3 expression was found to be significantly lower in all the ALL subtypes of leukaemia excluding the mature B-ALL t(8;14) patient samples when compared to the control group (figure 3.4). TRIB3 expression was also found to be lower in the AML t(11q23)/MLL, the AML with normal karyotype and other abnormalities and in the CLL patient samples when compared to the control group.

Performing the same analysis of TRIB1, TRIB2 and TRIB3 expression on the cells of haematopoiesis' revealed that there was a variation in TRIB1 (figure 3.1) and TRIB2 (figure 3.2) expression during haematopoiesis. This is in contrast to TRIB3 whose expression did not vary as widely between normal cells of the hematopoietic system (figure 3.4).

In the cells of haematopoiesis TRIB1 expression is highest in the monocytes and lowest in the T cell compartment (figure 3.1). Analyses of TRIB2 expression in the normal haematopoietic system found expression levels to be highest in the lymphocytes, with T cells having the highest relative expression across the hematopoietic system followed by the B cells and NKA cells (figure 3.2). Lowest TRIB2 expression is found in the monocytes, this is in direct contrast to TRIB1 whose expression is highest in this cellular subtype of the haematopoietic system. For TRIB3 expression levels are equivalent across the cellular compartments meaning that TRIB3 does not have the distinct cell specific expression of TRIB1 and TRIB2 in specific compartments of the hematopoietic system. The highest relative expression of TRIB3 is found in the granulocytes and the lowest expression is found in the eosinophils (figure 3.4).

Closer analyses of the TRIB1 and TRIB2 levels in the subpopulations of the T, B NK, Erythroid and dendritic cells revealed that TRIB1 and TRIB2 levels significantly vary as these cell types differentiate into their mature form (figures 3.5, 3.6, 3.7, 3.8 and 3.9). TRIB3 showed no significant differences in expression levels in these cellular subpopulations (appendix D figures D1, D2 and D3).

TRIB2 levels are highest in the T cells of all the cellular compartments of haematopoiesis (Figure 3.2). Closer analyses revealed that TRIB2, unlike TRIB1 or TRIB3, levels are significantly higher in the CD4+ T Helper cells compared to the CD8+ Cytotoxic T cells (figure 3.5). Looking at TRIB2 levels in the individual CD4+ T Helper cell populations revealed that TRIB2 levels in the CD4+ naïve, effector memory and central memory T cells

are significantly higher than TRIB2 levels in the CD8+ naïve, effector memory RA and effector memory T cells but are not significantly higher than the CD8+ central memory T cells (figure 3.5). While no significant difference in TRIB1 levels was found between the CD4+ and CD8+ T cells closer analysis revealed that TRIB1 levels in the CD4+ central memory T cells were significantly higher than those in the CD8+ naïve and central memory T cells (figure 3.5).

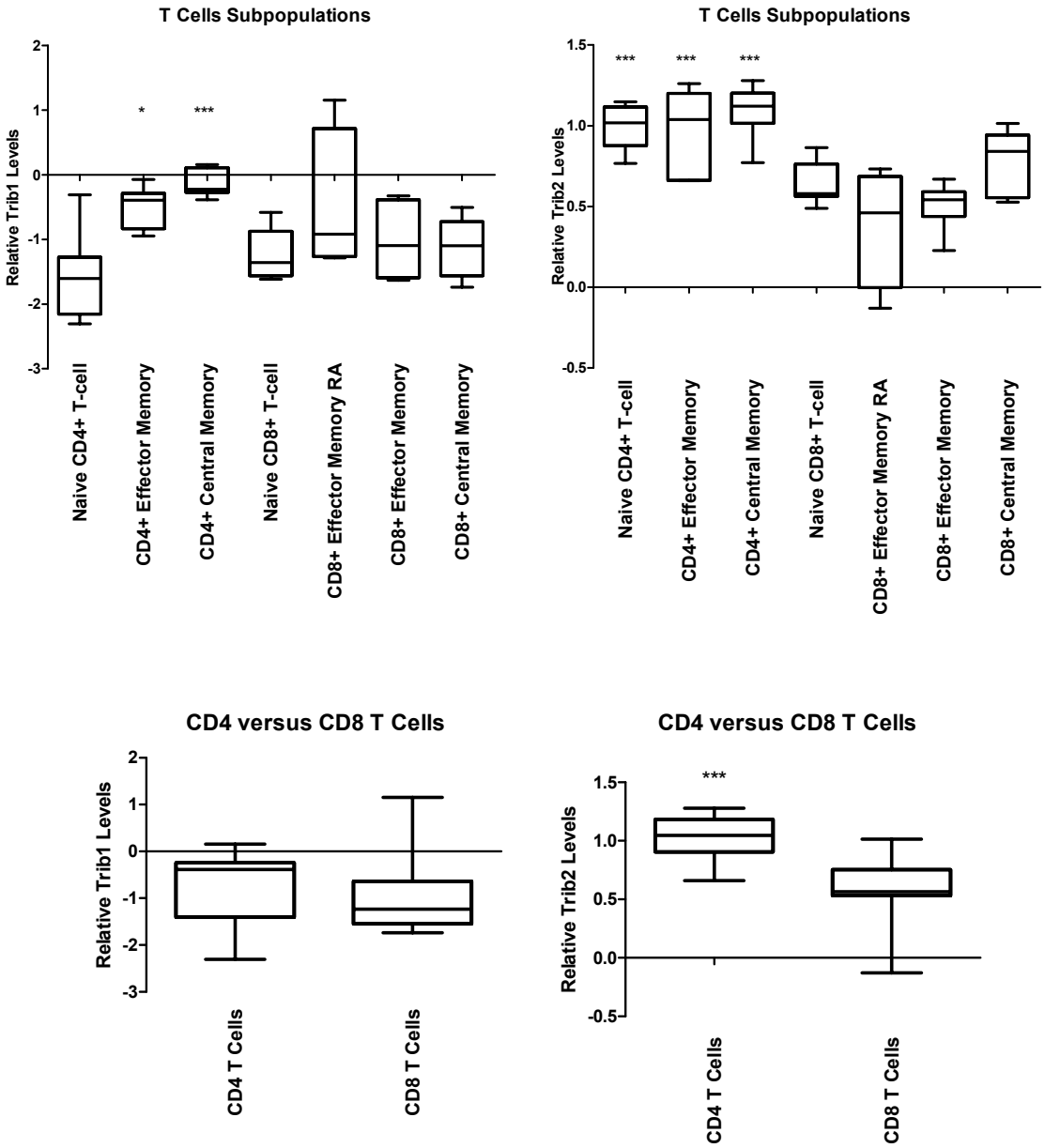

Figure 3.5: Expression profiles of TRIB1 and TRIB2 in T cell subpopulations. Samples are plotted as a boxes and whiskers graph. The box extends from the 25th to 75th percentiles and the line in the boxes represents the median value of the samples. The whiskers extend from the maximum to the minimum sample value. Statistical analyses were carried out by performing a one-way ANOVA analyses followed by a Bonferroni's Multiple Comparison Test of each of the cell types versus the others in graphs with more than two plots using GraphPad Prism 5. In those with two plots the student t-test was performed instead. A p-value below 0.05 indicated a significant difference in gene expression the cell types, p-value below 0.05 is represented by *, below 0.01 by ** and below 0.001 by ***. CD4 T Cells, N = 21; CD8 T Cells, N = 24; Naive CD4+ T-cell, N = 7; CD4+ Effector Memory, N = 7; CD4+ Central Memory, N = 7; Naive CD8+ T-cell, N = 7; CD8+ Effector Memory RA, N = 4; CD8+ Effector Memory, N = 6; CD8+ Central Memory, N = 7.

As the B cells mature TRIB1 and TRIB2 expression levels once again follow a follow a distinct and divergent pattern. While both TRIB1 and TRIB2 expression increases as the cells differentiate from early into pro B cells, TRIB1 expression, which is above the median, drops significantly in the naïve cells before increasing as the cells are activated and become mature B cells (figure 3.6). This is in contrast to TRIB2 levels which increase significantly below the median levels in the early and pro B cells to above the median in the naïve and mature B cells (figure 3.6). No significant changes in TRIB3 expression levels are observed (appendix D figure D2).

TRIB1 and TRIB2 expression levels in the NK cell populations follow a distinct and divergent pattern. TRIB1 expression is significantly lower in the mature cytotoxic NK cells and in the Mature CD56-CD16-CD3- NK cells compared to the precursor NK cells (figure 3.7). This is in direct contrast to TRIB2 expression where TRIB2 expression in the precursor NK cells is lower than the median and is significantly lower than that found in the mature cytotoxic NK cells and the Mature CD56-CD16-CD3- NK cells where TRIB2 expression levels are above the median (figure 3.7).

TRIB1 expression decreases but TRIB2 expression increases as the NK cell cells mature from their precursor form into cytotoxic NK cells. TRIB1 and TRIB2 expression also significantly varies in the NKT subpopulation compared to the other subpopulations of NK cell showing increased TRIB1 expression compared to the mature NK cells and decreased TRIB2 expression compared to the CD56-CD16-CD3- NK cells (figure 3.7). No significant differences in TRIB3 expression levels were found between the NK cell types (appendix D figure D3).

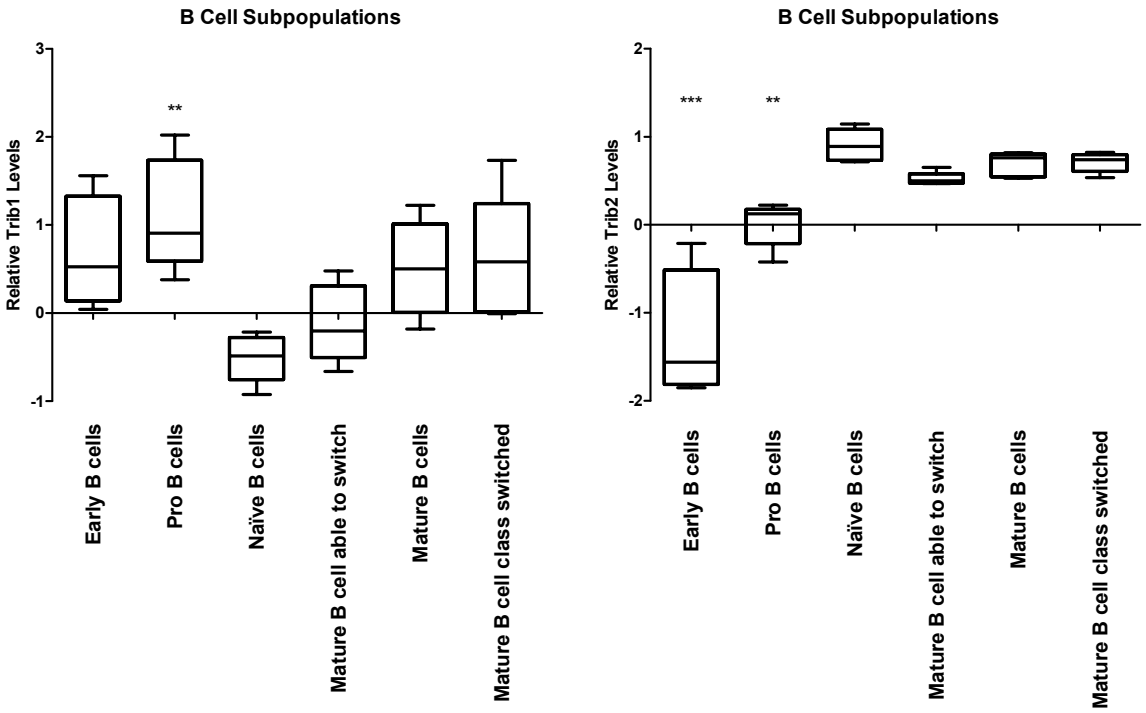

Figure 3.6: Expression profiles of TRIB1 and TRIB2 in the B subpopulations. Samples are plotted as a boxes and whiskers graph. The box extends from the 25th to 75th percentiles and the line in the boxes represents the median value of the samples. The whiskers extend from the maximum to the minimum sample value. Statistical analyses were carried out by performing a one-way ANOVA analyses followed by a Bonferroni's Multiple Comparison Test of each of the cell types versus the others in graphs with more than two plots using GraphPad Prism 5. In those with two plots the student t-test was performed instead. A p-value below 0.05 indicated a significant difference in gene expression the cell types, p-value below 0.05 is represented by *, below 0.01 by ** and below 0.001 by ***. Early B cells, N = 4; Pro B cells, N = 5; Naïve B cells, N = 5; Mature B cell able to switch, N = 5; Mature B cells, N = 5; Mature B cell class switched, N = 5.

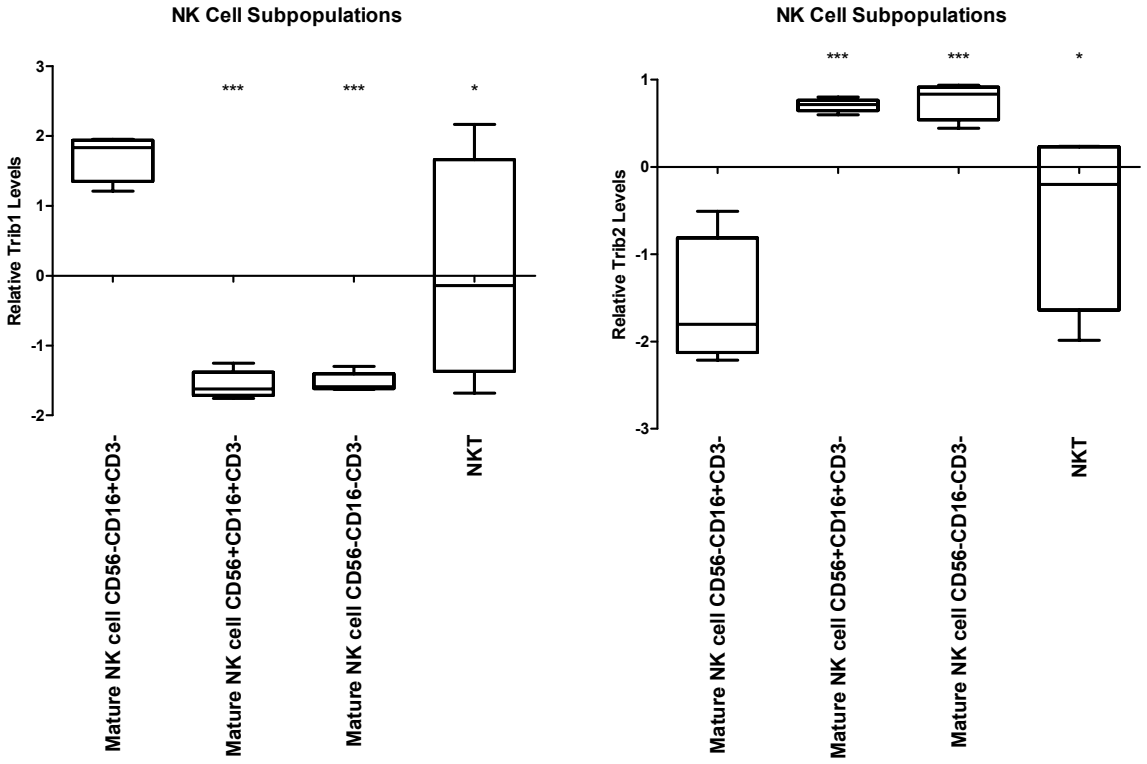

Figure 3.7: Expression profiles of TRIB1 and TRIB2 in the NK cell subpopulations. Samples are plotted as a boxes and whiskers graph. The box extends from the 25th to 75th percentiles and the line in the boxes represents the median value of the samples. The whiskers extend from the maximum to the minimum sample value. Statistical analyses were carried out by performing a one-way ANOVA analyses followed by a Bonferroni's Multiple Comparison Test of each of the cell types versus the others in graphs with more than two plots using GraphPad Prism 5. In those with two plots the student t-test was performed instead. A p-value below 0.05 indicated a significant difference in gene expression the cell types, p-value below 0.05 is represented by *, below 0.01 by ** and below 0.001 by ***. Mature NK cell CD56-CD16+CD3-, N = 4; Mature NK cell CD56+CD16+CD3-, N = 5; Mature NK cell CD56-CD16-CD3-, N = 5; NKT, N = 4.

In the erythroid cells both TRIB1 and TRIB2 follow a similar pattern of expression from increased levels in the early erythroid cells before a sharp and significant decrease in expression as the cells mature from CD34- CD71+ GlyA- erythroid cells into CD34- CD71+ GlyA+ erythroid cells before slowly rising again as the cells mature into CD34- CD71- GlyA+ erythroid cells. TRIB1 expression increases above the median in the CD34- CD71- GlyA+ erythroid cells but TRIB2 expression remains just below median expression levels (Figure 3.8). A drop in the levels of both TRIB1 and TRIB2 as the cells change from CD34- CD71+ GlyA- erythroid cells into CD34- CD71+ GlyA+ erythroid cells suggests that both may need to be decreased in order for the erythroid cells to mature.

Dendritic cells also show a similar pattern in TRIB1 and TRIB2 expression with significantly higher TRIB1 and TRIB2 levels observed in the myeloid compared to the plasmacytoid dendritic cells (figure 3.9). TRIB3 expression levels in both the erythroid and dendritic cells show no significant differences (appendix D figure D3).

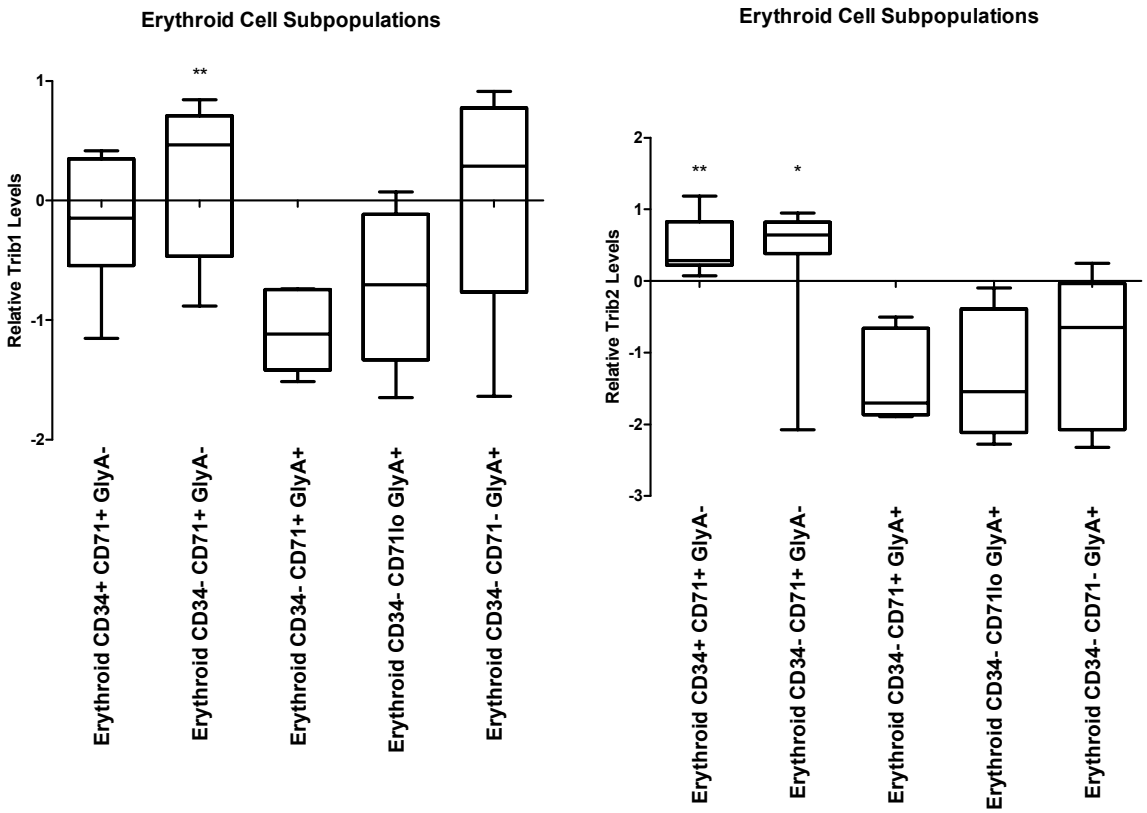

Figure 3.8: Expression profiles of TRIB1 and TRIB2 in erythroid cell subpopulations. Samples are plotted as a boxes and whiskers graph. The box extends from the 25th to 75th percentiles and the line in the boxes represents the median value of the samples. The whiskers extend from the maximum to the minimum sample value. Statistical analyses were carried out by performing a one-way ANOVA analyses followed by a Bonferroni's Multiple Comparison Test of each of the cell types versus the others in graphs with more than two plots using GraphPad Prism 5. In those with two plots the student t-test was performed instead. A p-value below 0.05 indicated a significant difference in gene expression the cell types, p-value below 0.05 is represented by *, below 0.01 by ** and below 0.001 by ***. Erythroid CD34+ CD71+ GlyA-, N = 7; Erythroid CD34- CD71+ GlyA-, N = 7; Erythroid CD34- CD71+ GlyA+, N = 6; Erythroid CD34- CD71lo GlyA+, N = 7; Erythroid CD34- CD71- GlyA+, N = 6.

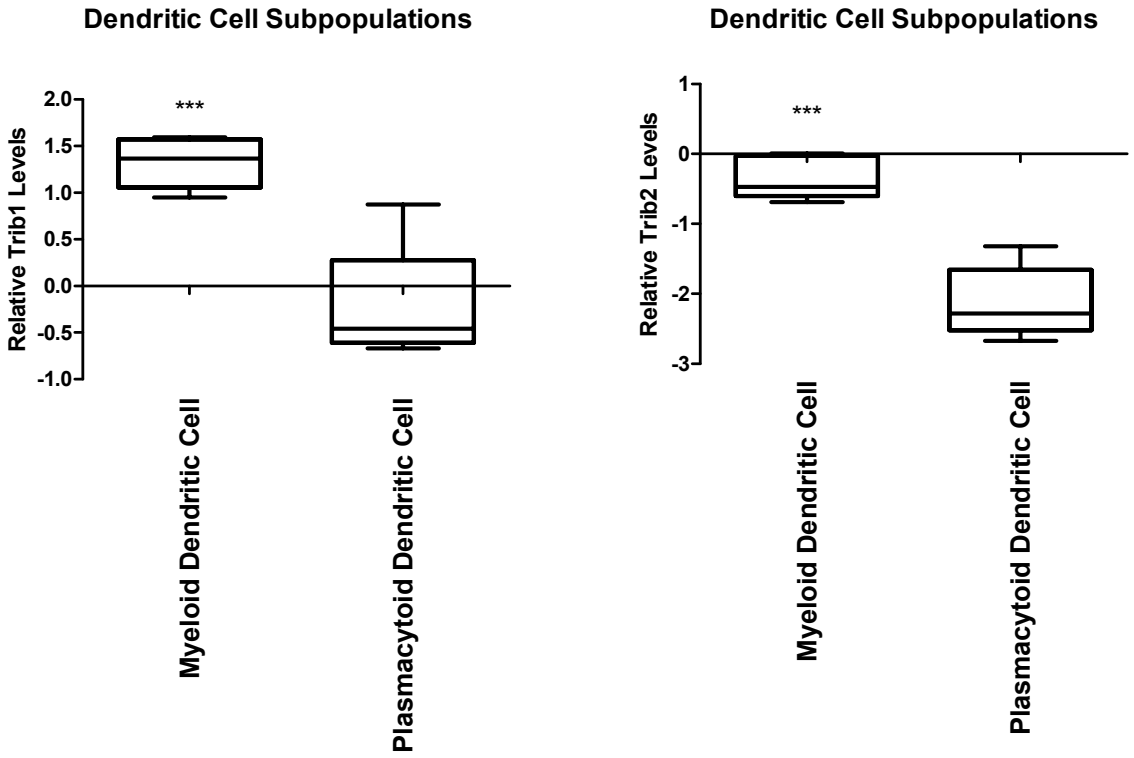

Figure 3.9: Expression profiles of TRIB1 and TRIB2 in dendritic cells subpopulations. Samples are plotted as a boxes and whiskers graph. The box extends from the 25th to 75th percentiles and the line in the boxes represents the median value of the samples. The whiskers extend from the maximum to the minimum sample value. Statistical analyses were carried out by performing a one-way ANOVA analyses followed by a Bonferroni's Multiple Comparison Test of each of the cell types versus the others in graphs with more than two plots using GraphPad Prism 5. In those with two plots the student t-test was performed instead. A p-value below 0.05 indicated a significant difference in gene expression the cell types, p-value below 0.05 is represented by *, below 0.01 by ** and below 0.001 by ***. N = 6; Myeloid Dendritic Cell, N = 5; Plasmacytoid Dendritic Cell, N = 5.

Analyses of TRIB1 and TRIB2 median centred levels in the subpopulations of the myeloid compartment revealed that TRIB1 and TRIB2 expression levels also vary in these cells as they mature into the granulocytes, monocytes, eosinophils, basophils and megakaryocytes (figures 3.10, 3.11, 3.12, 3.13 and 3.14)). Once again TRIB3 showed no significant differences in expression between the sub cellular populations of these cells (appendix D figure D4).

Enforced Trib2 expression in mice perturbs myeloid development; promoting monocyte and inhibiting granulocyte development (Keeshan et al., 2006). TRIB2 expression significantly changes during the development of both the granulocytic and monocytic cells in humans. In granulocytes TRIB2 expression rises as the cells mature from the HSCs into the mature granulocytes. TRIB2 expression is significantly up regulated in the colony forming unit granulocyte (CFU Granulocyte) cells, in the Granulocyte (Neutrophilic Metamyelocyte) (Granulocyte (NM)) cells and in the Granulocyte (Neutrophil) cells compared to the early CD133+ CD34dim hematopoietic stem cells (CD133+ CD34dim HSCs) (figure 3.10). During monocytic development TRIB2 expression, which rise as the cells mature from the early HSCs to the later GMP cells, is significantly down regulated as the cells differentiate into the mature monocytes. TRIB2 levels are significantly lower in the colony forming unit monocytes (CFU Monocytes) and in the mature monocytes compared to the earlier GMP cells and are also significantly lower in monocytes compared to the earlier CMP cells (figure 3.11). Down regulation of TRIB2 expression may be an important step in monocytic development.

In the basophils, eosinophils and megakaryocytes TRIB2 expression is increased in the mature cells compared to the early progenitor cells of haematopoiesis. TRIB2 levels in basophils are significantly higher in the mature basophils compared the CD133+ CD34dim and CD38- CD34+ HSCs (figure 3.12). TRIB2 levels in the mature eosinophils are

significantly higher than the CD133+ CD34dim HSCs (figure 3.13). Finally TRIB2 levels in the MEP cells, in the colony forming unit megakaryocytic (CFU megakaryocytic) cells and in the mature megakaryocytes is significantly higher than TRIB2 levels in the CD133+ CD34dim HSCs (figure 3.14). TRIB2 levels in the mature megakaryocytes are also significantly lower than levels in the MEP cells (figure 3.14).

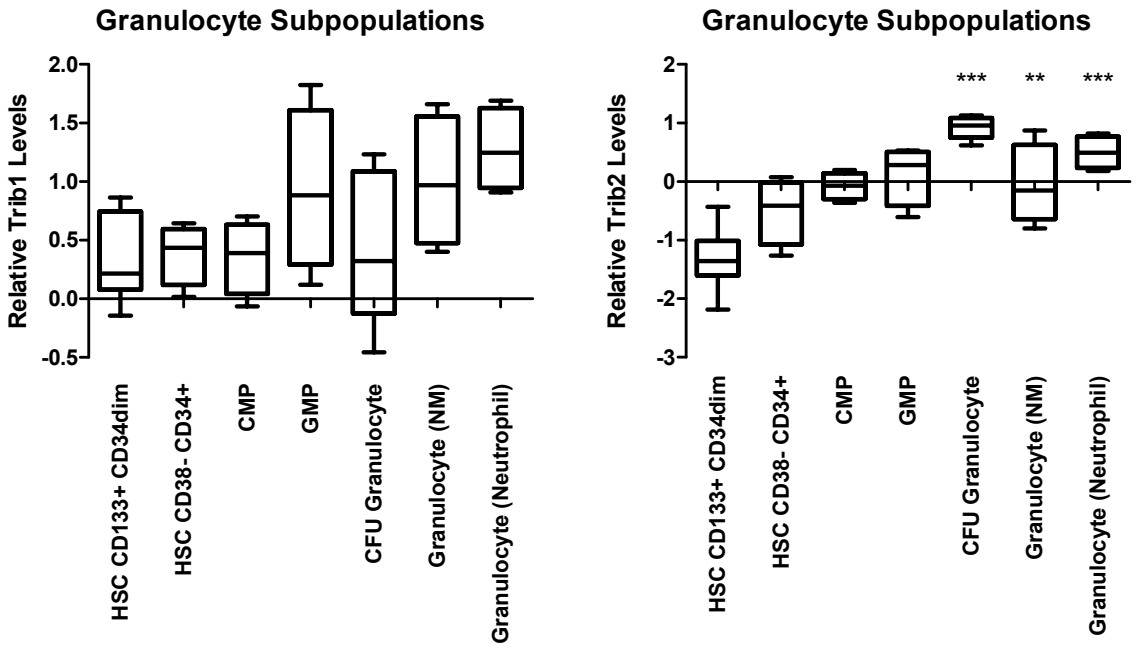

Figure 3.10: Expression profiles of TRIB1 and TRIB2 in Granulocyte Subpopulations. Samples are plotted as a boxes and whiskers graph. The box extends from the 25th to 75th percentiles and the line in the boxes represents the median value of the samples. The whiskers extend from the maximum to the minimum sample value. Statistical analyses was carried out by performing a one-way ANOVA analyses followed by a Bonferroni's Multiple Comparison Test of each of the cell types versus the others using GraphPad Prism 5. A p-value below 0.05 indicated a significant difference in gene expression the cell types, p-value below 0.05 is represented by *, below 0.01 by ** and below 0.001 by ***. HSC CD133+ CD34dim, N = 10; HSC CD38- CD34+, N = 4; CMP, N = 4; GMP, N = 4.

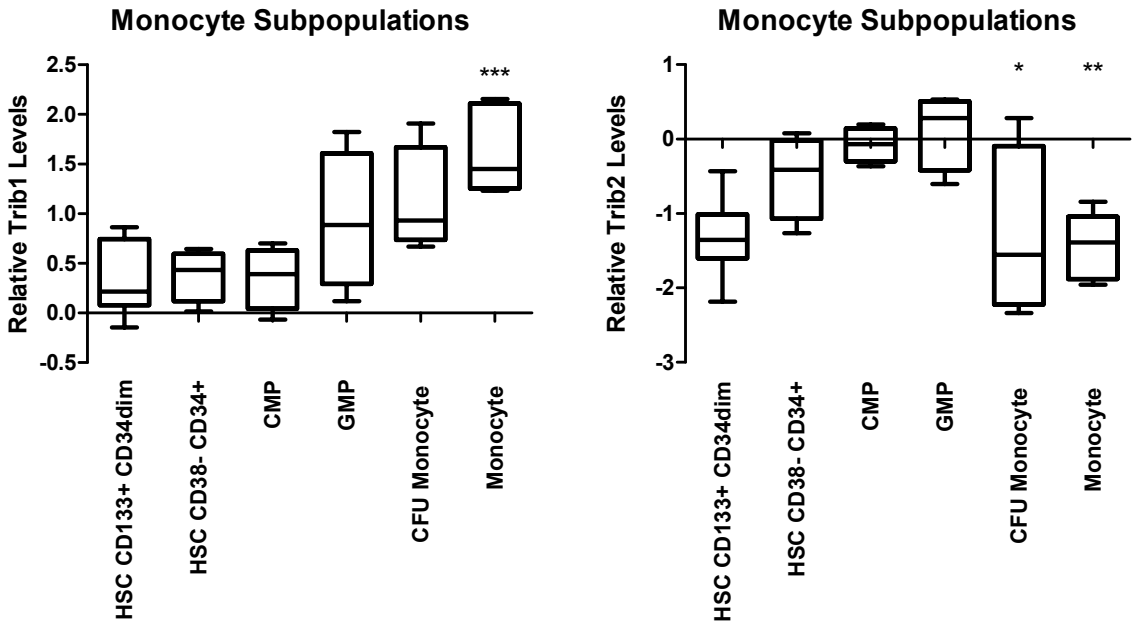

Figure 3.11: Expression profiles of TRIB1 and TRIB2 in Monocyte Subpopulations. Samples are plotted as a boxes and whiskers graph. The box extends from the 25th to 75th percentiles and the line in the boxes represents the median value of the samples. The whiskers extend from the maximum to the minimum sample value. Statistical analyses was carried out by performing a one-way ANOVA analyses followed by a Bonferroni's Multiple Comparison Test of each of the cell types versus the others using GraphPad Prism 5. A p-value below 0.05 indicated a significant difference in gene expression the cell types, p-value below 0.05 is represented by *, below 0.01 by ** and below 0.001 by ***. HSC CD133+ CD34dim, N = 10; HSC CD38- CD34+, N = 4; CMP, N = 4; GMP, N = 4; CFU Monocyte, N = 4; Monocyte, N = 5.

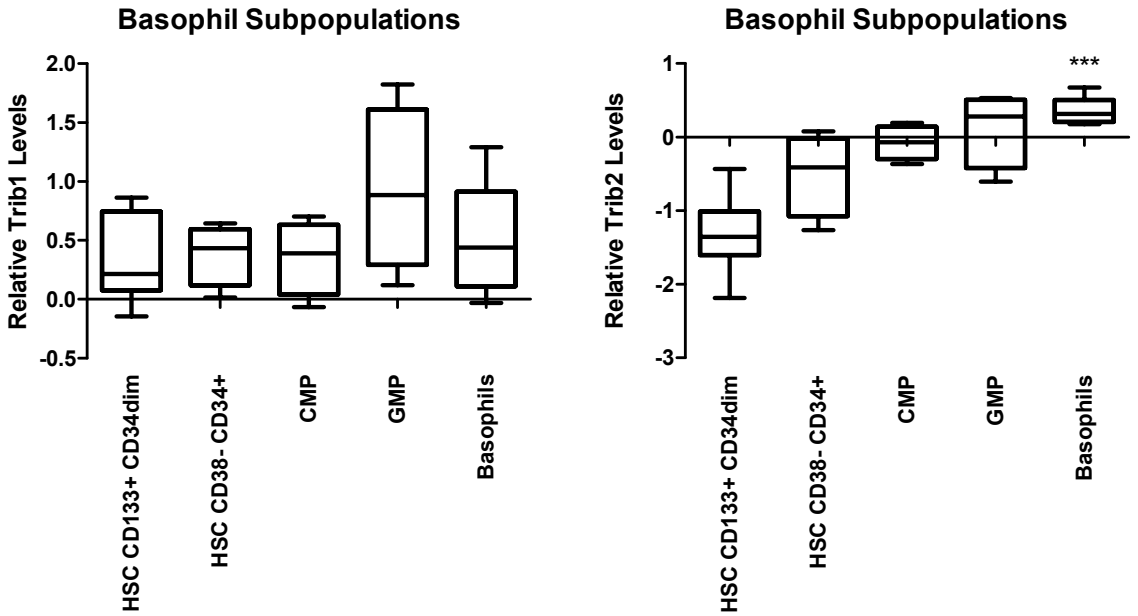

Figure 3.12: Expression profiles of TRIB1 and TRIB2 and TRIB3 expression in Basophil Subpopulations. Samples are plotted as a boxes and whiskers graph. The box extends from the 25th to 75th percentiles and the line in the boxes represents the median value of the samples. The whiskers extend from the maximum to the minimum sample value. Statistical analyses was carried out by performing a one-way ANOVA analyses followed by a Bonferroni's Multiple Comparison Test of each of the cell types versus the others using GraphPad Prism 5. A p-value below 0.05 indicated a significant difference in gene expression the cell types, p-value below 0.05 is represented by *, below 0.01 by ** and below 0.001 by ***. HSC CD133+ CD34dim, N = 10; HSC CD38- CD34+, N = 4; CMP, N = 4; GMP, N = 4; Basophils, N = 6.

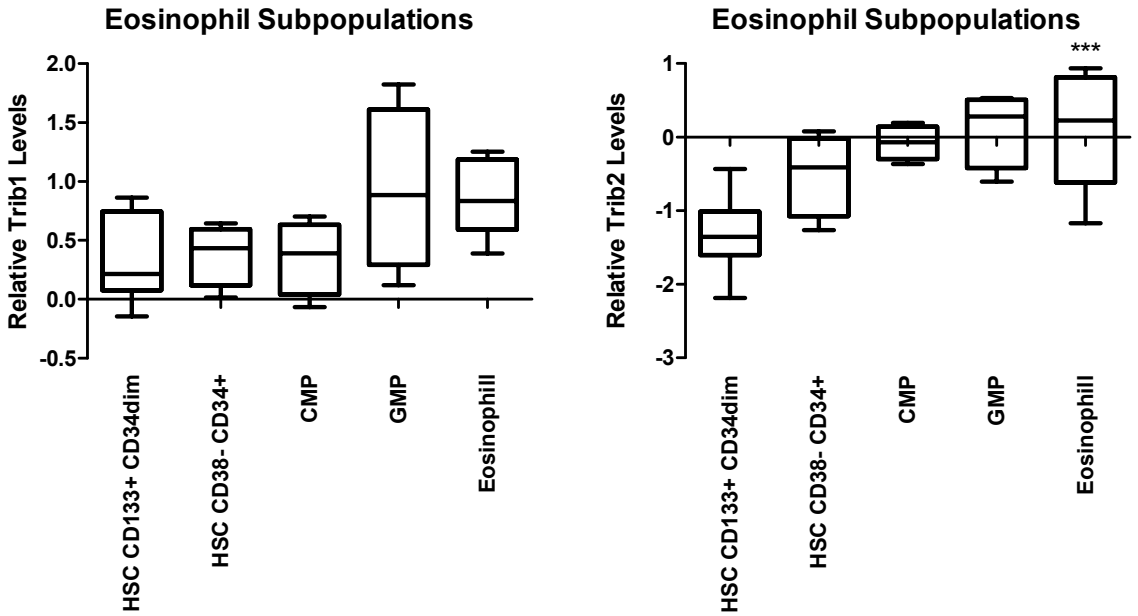

Figure 3.13: Expression profiles of TRIB1 and TRIB2 in Eosinophil Cell. Samples are plotted as a boxes and whiskers graph. The box extends from the 25th to 75th percentiles and the line in the boxes represents the median value of the samples. The whiskers extend from the maximum to the minimum sample value. Statistical analyses was carried out by performing a one-way ANOVA analyses followed by a Bonferroni's Multiple Comparison Test of each of the cell types versus the others using GraphPad Prism 5. A p-value below 0.05 indicated a significant difference in gene expression the cell types, p-value below 0.05 is represented by *, below 0.01 by ** and below 0.001 by ***. HSC CD133+ CD34dim, N = 10; HSC CD38- CD34+, N = 4; CMP, N = 4; GMP, N = 4; Eosinophill, N = 5.

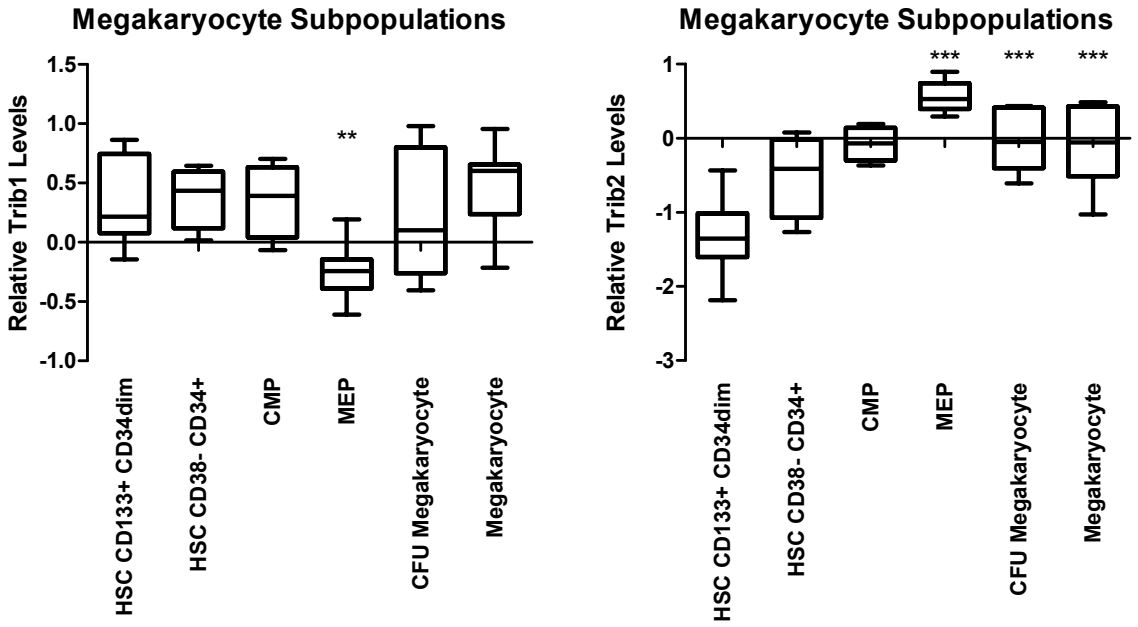

Figure 3.14: Expression profiles of TRIB1 and TRIB2 in Megakaryocyte subpopulations. Samples are plotted as a boxes and whiskers graph. The box extends from the 25th to 75th percentiles and the line in the boxes represents the median value of the samples. The whiskers extend from the maximum to the minimum sample value. Statistical analyses was carried out by performing a one-way ANOVA analyses followed by a Bonferroni's Multiple Comparison Test of each of the cell types versus the others using GraphPad Prism 5. A p-value below 0.05 indicated a significant difference in gene expression the cell types, p-value below 0.05 is represented by *, below 0.01 by ** and below 0.001 by ***. HSC CD133+ CD34dim, N = 10; HSC CD38- CD34+, N = 4; CMP, N = 4; MEP, N = 9; CFU Megakaryocyte, N = 5; Megakaryocyte, N = 7.

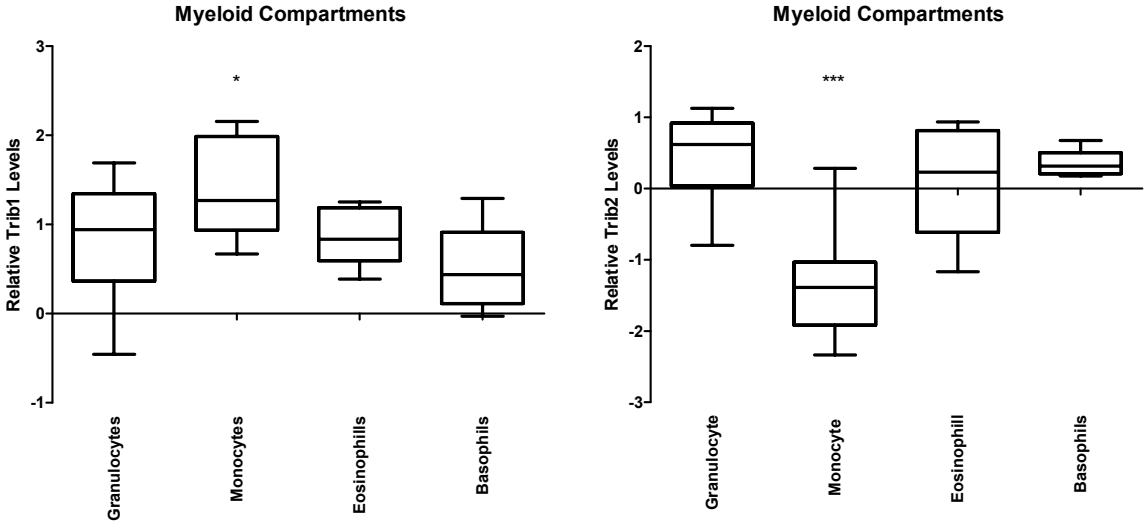

Figure 3.15: Expression profiles of TRIB1 and TRIB2 in the granulocyte, monocyte, basophile and eosinophil cellular compartments. Samples are plotted as a boxes and whiskers graph. The box extends from the 25th to 75th percentiles and the line in the boxes represents the median value of the samples. The whiskers extend from the maximum to the minimum sample value. Statistical analyses was carried out by performing a one-way ANOVA analyses followed by a Bonferroni's Multiple Comparison Test of each of the cell types versus the others using GraphPad Prism 5. A p-value below 0.05 indicated a significant difference in gene expression the cell types, p-value below 0.05 is represented by *, below 0.01 by ** and below 0.001 by ***. Basophils, N = 6; Eosinophill, N = 5; MEP, N = 9; Granulocytes, N = 13; Monocytes, N = 9.

TRIB1 expression increases as both the granulocytes and the monocytes mature, but only in the monocytes is this increase significant (figures 3.10 and 3.11). The mature monocytes possess the highest levels of TRIB1 expression compared to all the other cellular compartments of haematopoiesis (figure 3.1)). TRIB1 levels in the mature monocytes are significantly higher than levels in the CD133+ CD34dim and CD38- CD34+ HSCs and in the CMP cells (figure 3.11). No significant changes in TRIB1 expression occurs during basophil or eosinophil expression though TRIB1 is significantly down regulated in the MEP cells compared to the CD133+ CD34dim HSCs during megakaryocyte development before increasing in the later megakaryocyte cells (figures 3.12, 3.13 and 3.14).

Comparison of overall TRIB1, TRIB2 and TRIB3 levels in the granulocytes, monocytes, eosinophils and basophils revealed that TRIB1 expression levels are significantly higher in the monocyte cells compared to the basophils while, in contrast, TRIB2 expression levels in the monocytes is significantly lower in the monocytes compared to the basophils, eosinophils and granulocytes (figure 3.15). TRIB3 levels, which do not significantly vary across the cellular subpopulations of haematopoiesis are significantly higher in the granulocytes compared to the monocytes or eosinophiles when only the mature cells of these compartments are analysed (appendix D figure D4).

The TRIB1 and TRIB2 of the Tribble gene family vary significantly between the cellular compartments and the sub cellular populations of the cells of haematopoiesis during cellular differentiation. Control of TRIB1 and TRIB2 expression is needed for the correct development of many of the haematopoietic cells as evidenced by the fact that elevated TRIB1 and TRIB2 expression leads to perturbed haematopoiesis and leukaemia (Keeshan et al., 2006; Jin et al., 2007; Dedhia et al., 2010). Both TRIB1 and TRIB2 levels vary during myeloid differentiation (figures 3.10 and 3.11) but expression analyses suggest that both TRIB1 and TRIB2 play a role in lymphoid differentiation (figures 3.5, 3.6 and 3.7). The

expression of TRIB1 and TRIB2 often follows an opposing pattern in both the cellular (figure 3.1 and 3.2) and sub cellular compartments of haematopoiesis as evidenced by both the B cell (figure 3.6) and monocyte cells (figure 3.11). Expression of TRIB3, which does not cause AML (Dedhia et al., 2010), does not vary significantly during haematopoietic development (appendix D figures D1, D2, D3 and D4), though TRIB3 levels are significantly higher in terminally differentiated granulocytes compared to mature monocytes, eosinophils and basophils only (appendix D, figure D4), indicating that TRIB3 expression does not affect haematopoiesis but does vary between the mature myeloid cells.

3.2 Identification and clustering of TRIB1, TRIB2 and TRIB3 gene neighbours in leukaemia and the cells of normal haematopoiesis

Using one-way ANOVA analyses the top differentially expressed genes between a high and low TRIB1, TRIB2 or TRIB3 signature was determined for AML, ALL, CML, CLL, MDS and AML with normal karyotype and other abnormalities patient samples of the MILE study. This lead to the creation of a gene signature for the TRIB1, 2 and 3 genes in these disease states made up of these top differentially expressed genes for each of the high versus low Tribble gene signatures in the above leukaemic types. Unsupervised hierarchical clustering of these gene neighbours resulted in the clustered of the patient samples based on these TRIB1, TRIB2 or TRIB3 gene signatures (figures 3.16, 3.17 and 3.18 and appendix D figures D5, D6, D7, D8, D9 and D10) thereby identifying patients clusters with leukaemia that possess a distinct TRIB1, TRIB2 or TRIB3 signature.

Clustering of the TRIB1 gene signature in each of these disease states in the MILE study revealed that a number of AML with normal karyotype and other abnormalities patient samples cluster together with increased TRIB1 expression (figure 3.16). No distinct cluster of

patient samples was evident in the ALL or total AML patient samples found in the MILE study (appendix D figure D5). The TRIB1 gene signature was not strongly associated with any specific AML or ALL leukaemia subtype. In the CLL and CML samples clustering TRIB1 and its gene neighbours expression does not reveal any distinct subset of patient samples that cluster together with either high or low TRIB1 gene expression (appendix D figure D6). However in the MDS patient samples a number of patient samples with high TRIB1 and gene neighbour expression do strongly cluster together indicating that a subgroup of MDS patient samples may possess a TRIB1 signature (appendix D figure D6).

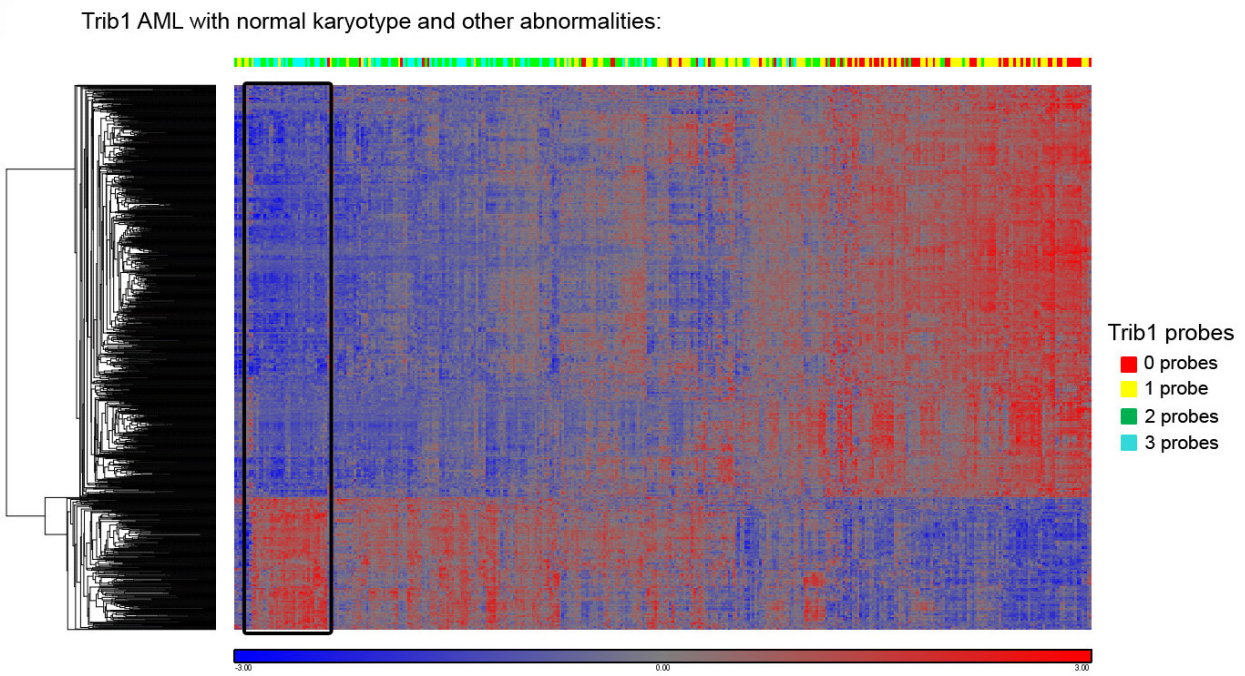

Figure 3.16: Heatmaps of TRIB1 and its gene neighbours clustered based on TRIB1 expression in the AML with normal Karyotype and other abnormalities. To determine the nearest-neighbours of TRIB1 each patient sample was separated based on whether TRIB1 expression was below the median expression of each of the three probe for TRIB1 in the microarray (labelled 0) or above the median expression in one (labelled 1), two (labelled 2) or three (labelled 3) of the probes sets. A one-way ANOVA analysis was then performed to determine genes with significantly different expression between the 0 and 3 group (below versus above the median for all three probe sets). Unsupervised hierarchical clustering of the top 1164 differentially expressed genes with a p-value of 0.00000000000544733 or less as determined by the ANOVA analysis was then performed using PARTEK GENOMICS SUITE (Version 6.6). See supplementary table 3.I for gene neighbours lists used to generate heatmaps on the accompanying CD.

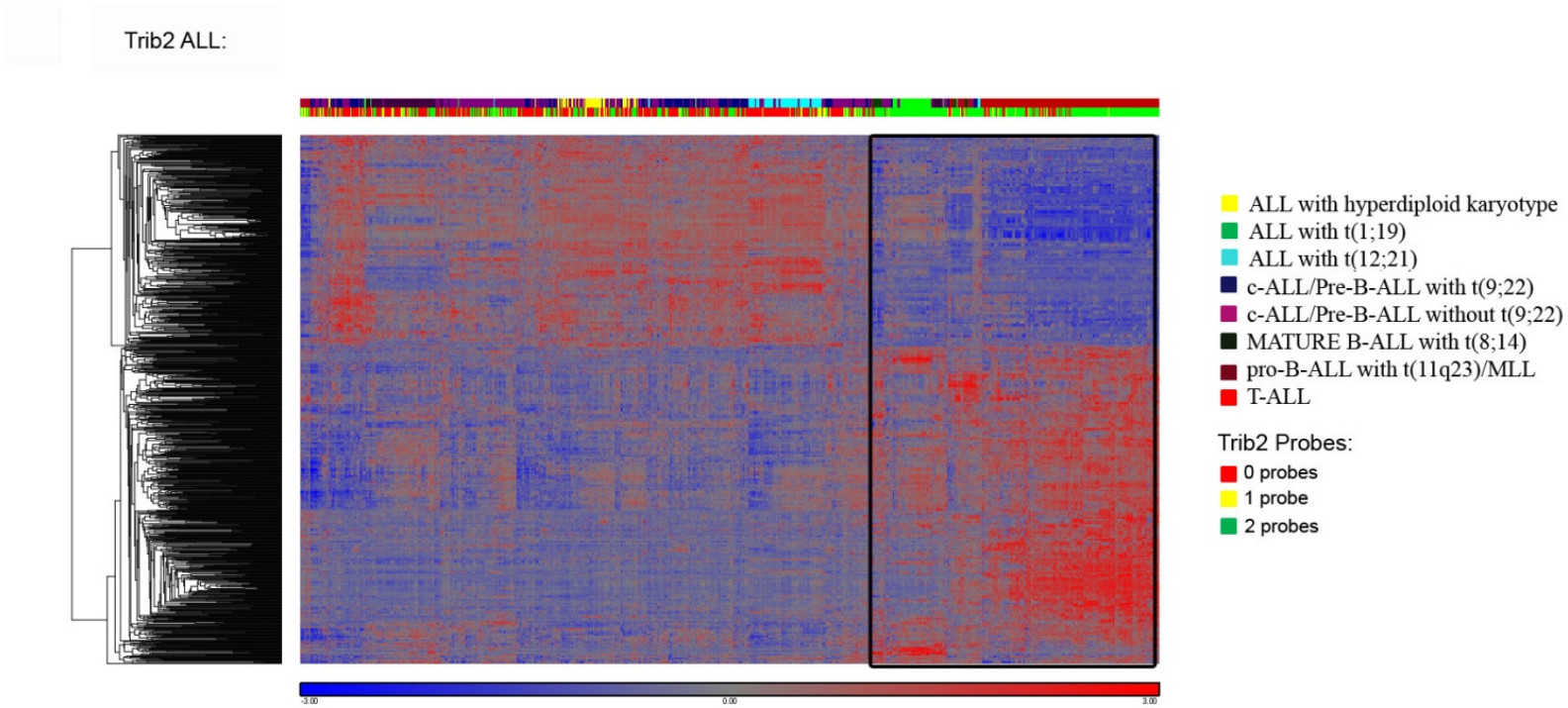

Figure 3.17: Heatmaps of TRIB2 and its gene neighbours clustered based on TRIB2 expression in the ALL patients samples of the MILE study. To determine the nearest-neighbours of TRIB2 in the MILE dataset for ALL each patient sample was separated based on whether TRIB2 expression was below the median expression of each of the two probe for TRIB2 in the microarray (labelled 0) or above the median expression in one (labelled 1) or two (labelled 2) of the probes sets. A one-way ANOVA analysis was then performed to determine genes with significantly different expression between the 0 and 2 group (below versus above the median for both probe sets). Unsupervised hierarchical clustering of the top 1003 differentially expressed genes with a p-value of 0.0000000000000839277 or less as determined by the ANOVA analysis was then performed using PARTEK GENOMICS SUITE (Version 6.6) for the ALL samples. See supplementary table 3.II for gene neighbours lists used to generate heatmaps on the accompanying CD

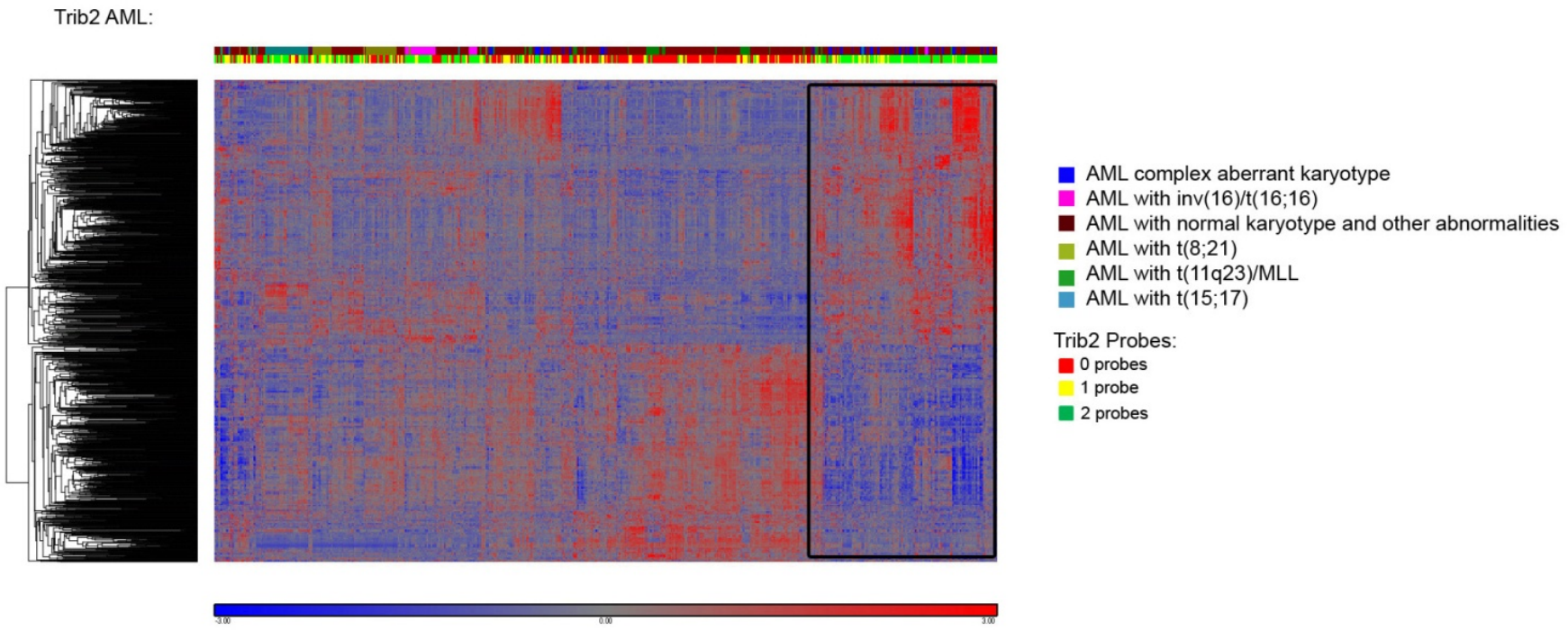

Figure 3.18: Heatmaps of TRIB2 and its gene neighbours clustered based on TRIB2 expression in the AML patients samples of the MILE study. To determine the nearest-neighbours of TRIB2 in the MILE dataset for AML each patient sample was separated based on whether TRIB2 expression was below the median expression of each of the two probe for TRIB2 in the microarray (labelled 0) or above the median expression in one (labelled 1) or two (labelled 2) of the probes sets. A one-way ANOVA analysis was then performed to determine genes with significantly different expression between the 0 and 2 group (below versus above the median for both probe sets). Unsupervised hierarchical clustering of the top 1351 differentially expressed genes with a p-value of 0.000000122264 or less as determined by the ANOVA analysis then performed using PARTEK GENOMICS SUITE (Version 6.6) for the AML samples. See supplementary table 3.II for gene neighbours lists used to generate heatmaps on the accompanying CD

Clustering of TRIB2 and its gene neighbours revealed that these gene cluster with the T-ALL and ALL with t(1;19) subtypes of ALL (Figure 3.17). This data was published in the British Journal of Haematology paper "Elevated TRIB2 with NOTCH1 activation in paediatric/adult T-ALL" (see appendix A).In the AML samples high TRIB2 expression and its top gene neighbours clustered with a mixture of AML with normal karyotype and other abnormalities and AML with complex aberrant karyotype (Figure 3.18). When only the AML with normal karyotype and other abnormalities samples were clustered based on TRIB2 expression, a small number of patient samples do cluster together with above the median TRIB2 expression forming a distinct subgroup of patient samples with high TRIB2 expression (appendix D figure D7).

For the CLL samples clustering of TRIB2 and its gene neighbours expression does not reveal any distinct subset of patient samples. CML had only eight statistically significant differentially expression genes between the high and low TRIB2 expressing samples compared to the approximately 1000 genes used to analyse the other leukaemia types. This indicates that there is little significant difference in gene expression between TRIB2 expressions in CML. The MDS samples clustering TRIB2 and its gene neighbours expression revealed a small cluster of patient samples associated with high TRIB2 and its gene neighbours indicating that a small number of MDS patient samples may possess a TRIB2 signature (appendix D figure D8).

In the ALL samples high TRIB3 and its top gene neighbours expression clustered with the T-ALL (appendix D figure D9). As with TRIB1 and 2, the AML samples high TRIB3 expression clustered with a mixture of AML with normal karyotype and other abnormalities and AML with complex aberrant karyotype (appendix D figure D9). When only the AML with normal karyotype and other abnormalities samples were clustered based on TRIB3 expression, a small amount of patient samples do cluster together with above the median

TRIB3 expression forming a distinct subgroup of patient samples with high TRIB3 expression (appendix D figure D9). In the CLL, CML and MDS samples there are also a small amount of patient samples that cluster together with above the median TRIB3 expression forming a distinct subgroup of patient samples with high TRIB3 expression (appendix D figure D10). This indicates that these leukaemia subtypes do possess subgroups with a distinct TRIB3 signature.

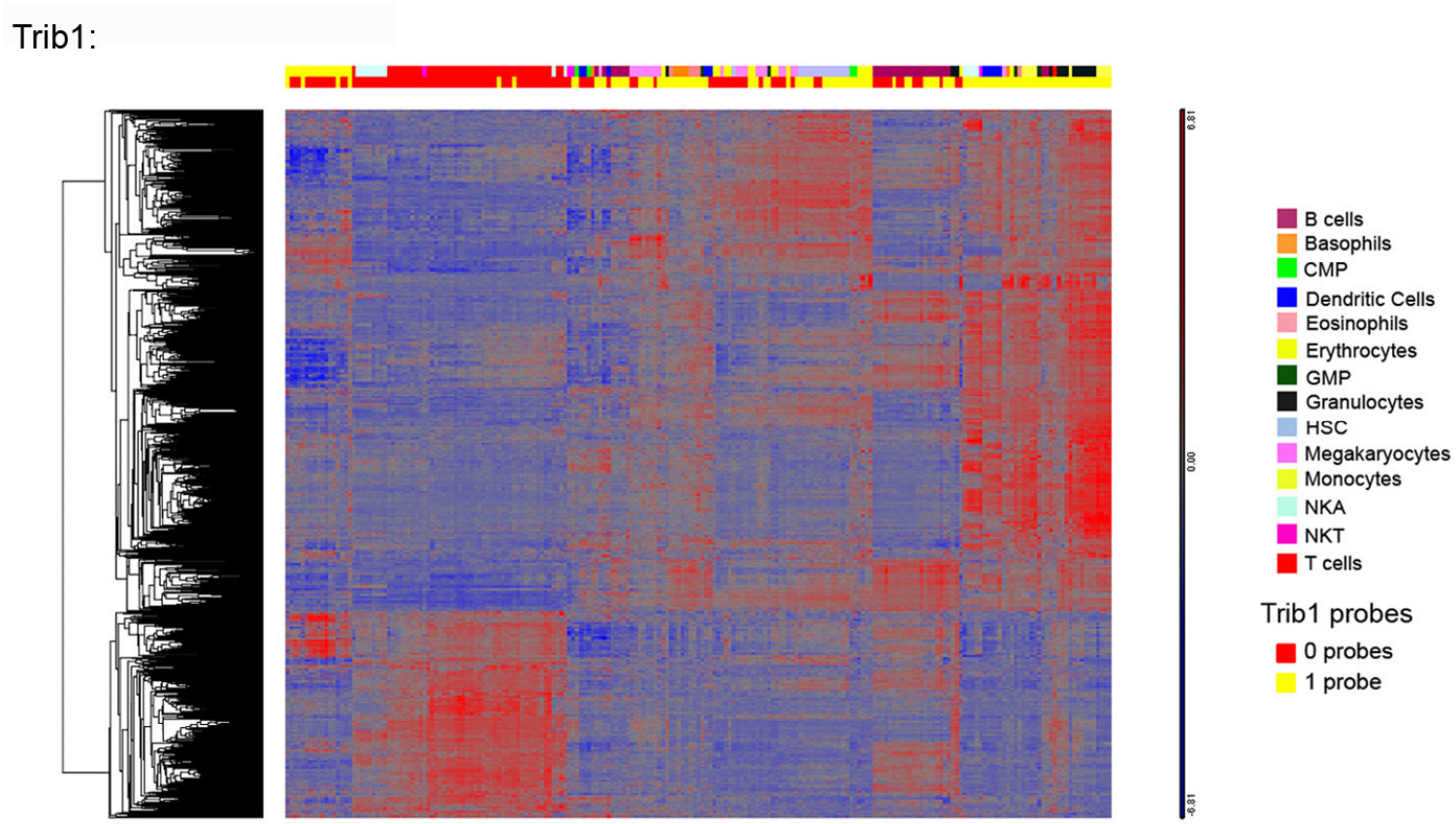

Figure 3.19: Heatmap of TRIB1 gene neighbours clustered based on TRIB1 expression in the cells of the haematopoietic system. To determine the nearest-neighbours of TRIB1 each sample was separated based on whether TRIB1 expression was below the median expression (labelled 0) or above the median expression (labelled 1) in its relevant probe. A one-way ANOVA analysis was then performed to determine genes with significantly different expression between the 0 and 1 group (below versus above the median for the probe sets). Unsupervised hierarchical clustering of the top 1343 differentially expressed genes with a p-value of 0.00000289779 or less as determined by the ANOVA analysis was then performed using PARTEK GENOMICS SUITE (Version 6.6). See supplementary table 3.IV for gene neighbours lists used to generate heatmaps on the accompanying CD.

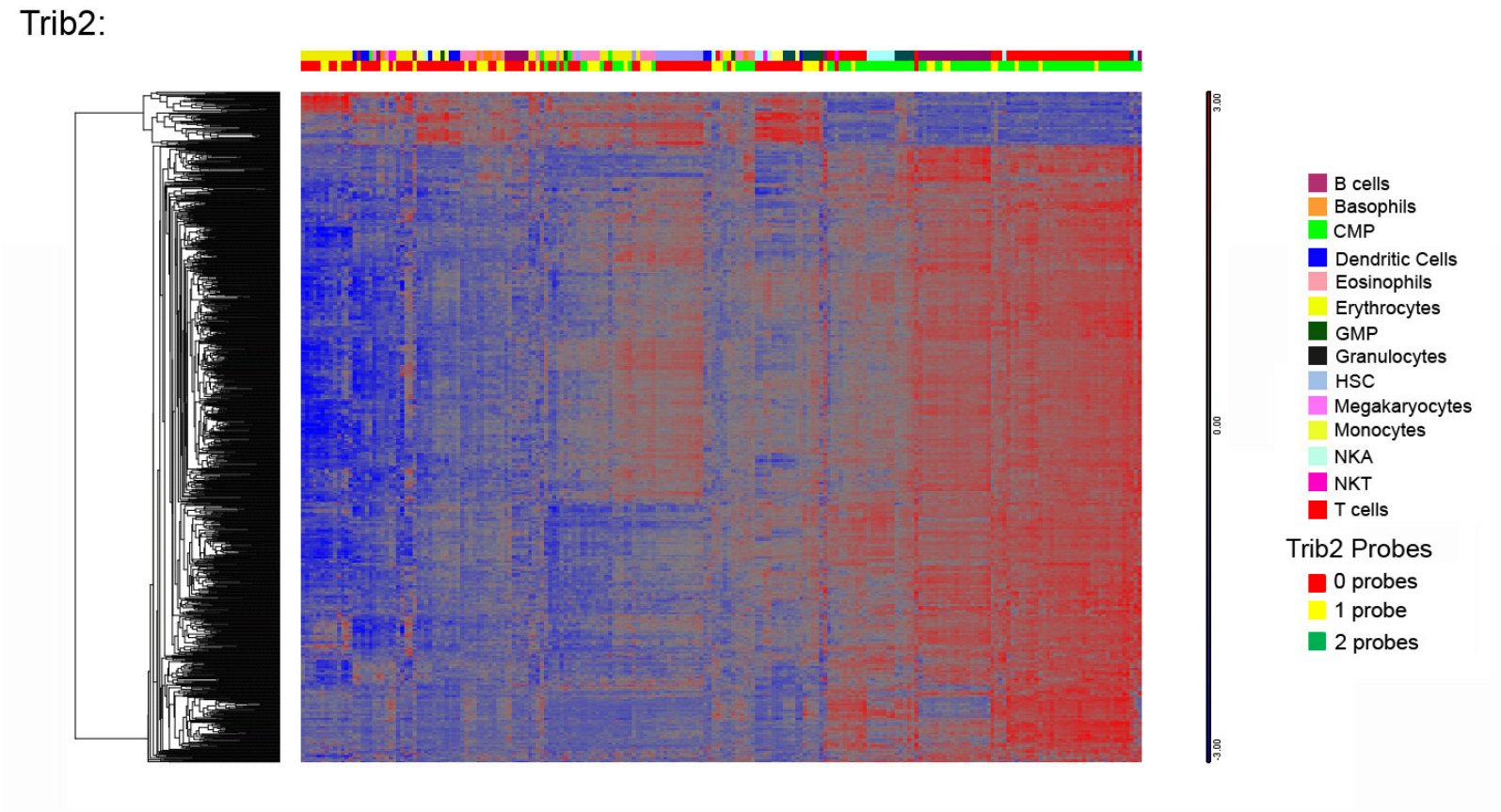

Figure 3.20: Heatmap of TRIB2 and its gene neighbours clustered based on TRIB2 expression in the cells of the haematopoietic system. To determine the nearest-neighbours of TRIB2 each sample was separated based on whether TRIB2 expression was below the median expression of each of the two probe for TRIB2 in the microarray (labelled 0) or above the median expression in one (labelled 1) or two (labelled 2) of the probes sets. A one-way ANOVA analysis was then performed to determine genes with significantly different expression between the 0 and 2 group (below versus above the median for the probe sets). Unsupervised hierarchical clustering of the top 1009 differentially expressed genes with a p-value of 0.000000000000188197 or less as determined by the ANOVA analysis was then performed using PARTEK GENOMICS SUITE (Version 6.6). See supplementary table 3.IV for gene neighbours lists used to generate heatmaps on the accompanying CD.

Clustering of TRIB1, TRIB2 and TRIB3 and their gene neighbours in the cells of the haematopoietic system as determined by Novershtern et al. show that low TRIB1 and its gene neighbours expression clusters primarily with the T cell samples (Figure 3.18). This is in direct contrast with TRIB2 expression, as high TRIB2 expression clusters with the T cell along with the B cell and NKA cell samples (Figure 3.19). High TRIB1 expression clusters with a mixture of cell types including the Granulocytes and Monocytes. No distinct cell type clusters primarily with high or low TRIB3 expression (appendix D figure D11). These analyses reveal that in the cells of haematopoiesis a TRIB1 signature associates with the monocytes and a TRIB2 signature associates with lymphocytes.

3.3 GSEA analyses of the TRIB1 and TRIB2 signature in the leukaemic subtypes of the MILE study using the Haematopoietic gene modules

Novershtern et al. determined that the human cells of haematopoiesis contained 80 modules of strongly co-expressed genes. These gene modules were classified by Novershtern et al. based on the cell type or types that they are expressed in. For example module numbered 673 is expressed in both T and B cell and is therefore classified as a T and B cell associated module. Using these gene modules GSEA analyses were performed for the TRIB1 and TRIB2 signature in the various leukaemic subtypes identified in the MILE study. These analyses were able to identify if the TRIB1 and TRIB2 signatures are enriched within specific cell types of the haematopoietic system.

Analyses of the TRIB1 signature identified enrichment for gene clusters or modules that are highly expressed in the late myeloid cells (Late MYE) in the majority of the leukaemic subtypes of the MILE study (no gene clusters were enriched for mature B-ALL with t(8;14), in the ALL with t(12;21), ALL with hyperdiploid karyotype, in the MDS or in the control group samples) (figures 3.20 and 3.21). Gene modules that are highly expressed in other cell types are also enriched in some of the leukaemic subtypes for the TRIB1 signatures. These include a module of genes highly expressed in HSC and early myeloid cells (HSE + Early Mye) AML with normal karyotype and other abnormalities samples for the TRIB1 signature. A module of genes that are highly expressed across the late erythrocytes, the T and B cells and the granulocytes (Late Ery + T/B-cell +GRAN) is also enriched for the TRIB1 signature in CLL patient samples.

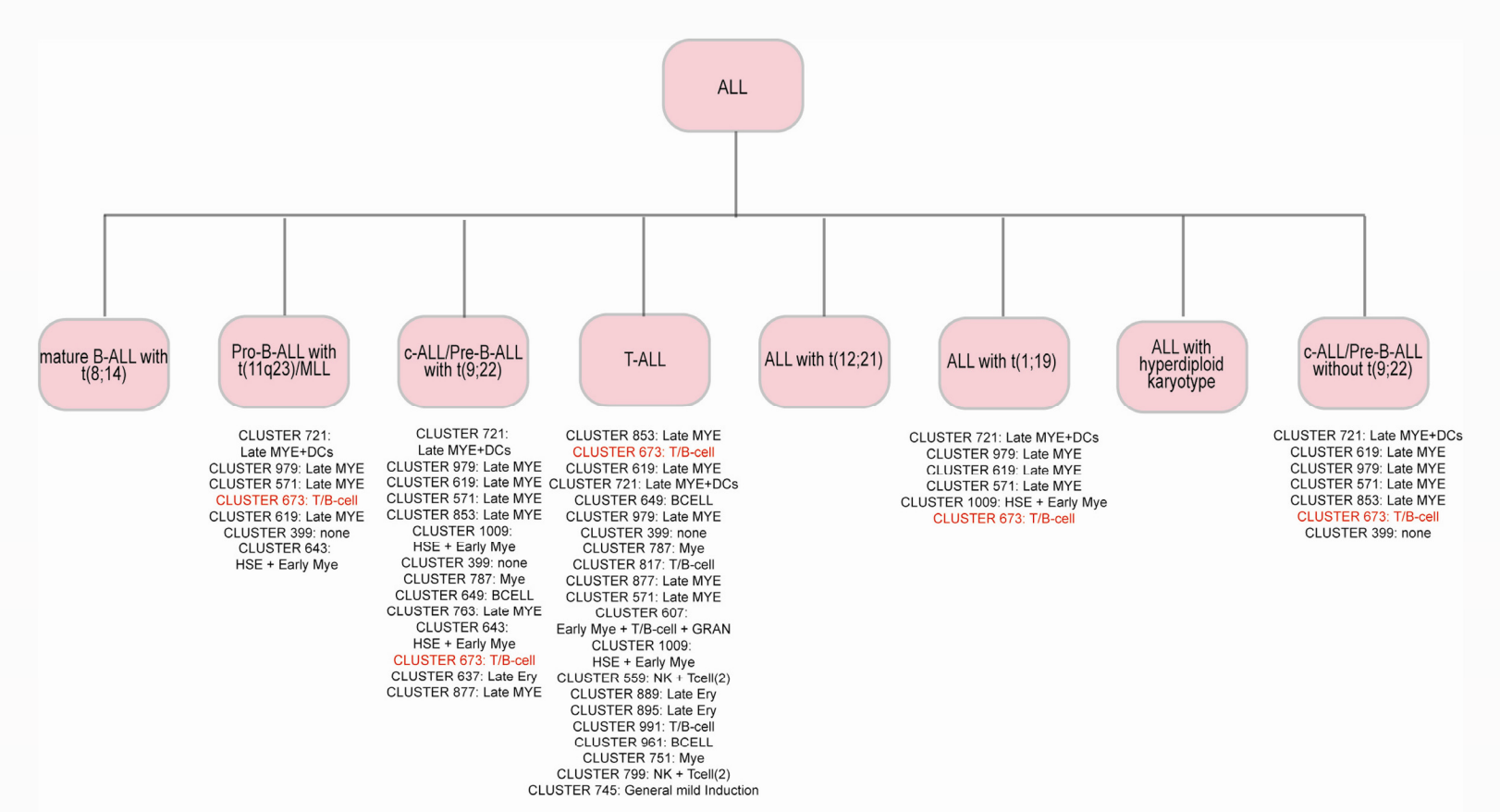

Figure 3.20: The haematopoietic gene modules enriched in ALL subsets of the MILE Study for the TRIB1 signature. GSEA was carried out for the TRIB1 signature in all the leukaemic subtypes of the MILE study. GSEA was run using the haematopoietic gene modules, 80 modules of strongly co-expressed genes identified by Novershtern at al. in the haematopoietic cell lineages (Novershtern et al., 2011). Each module of genes was classified based on where the genes of each gene module were expresses within the haematopoietic cell lineage. Enrichment for individual gene modules was determined for the TRIB1 signature for each of the leukaemic subtypes of the MILE study. Enriched gene module type can be seen listed in the above figure below the relevant leukaemia subtype ranked in order of negative enrichment score (NES). Detailed tables with names of individual pathways enriched and p-values, q-value and NES can be found in the supplementary table 3.V on the accompanying CD.

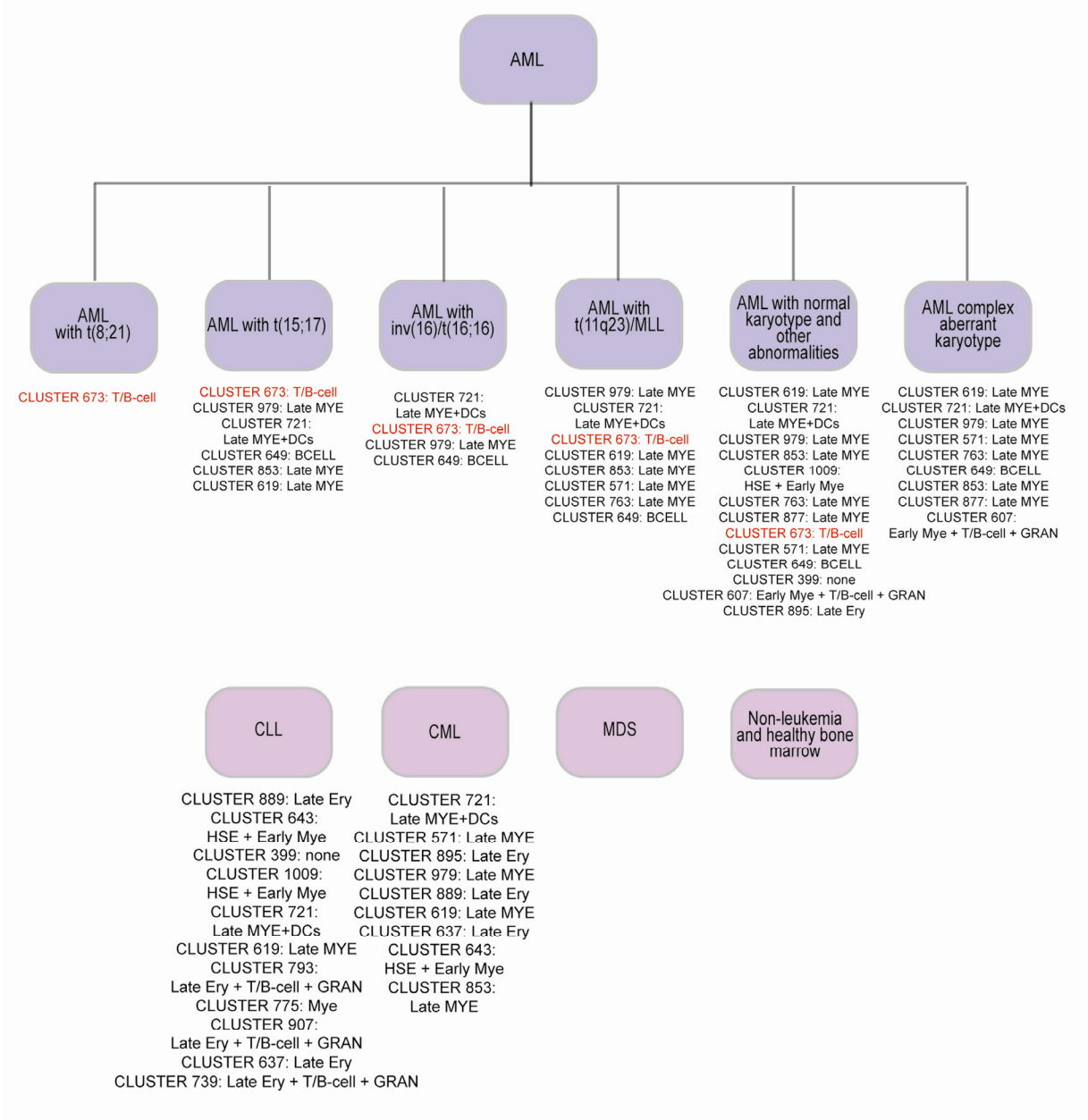

Figure 3.21: The haematopoietic gene modules enriched in AML subsets, CLL, CML, MDS and Non-leukaemic control group of the MILE Study for the TRIB1 signature. GSEA was carried out for the TRIB1 signature in all the leukaemic subtypes of the MILE study. GSEA was run using the haematopoietic gene modules, 80 modules of strongly co-expressed genes identified by Novershtern at al. in the haematopoietic cell lineages (Novershtern et al., 2011). Each module of genes was classified based on where the genes of each gene module were expresses within the haematopoietic cell lineage. Enrichment for individual gene modules was determined for the TRIB1 signature for each of the leukaemic subtypes of the MILE study. Enriched gene module type can be seen listed in the above figure below the relevant leukaemia subtype ranked in order of negative enrichment score (NES). Detailed tables with names of individual pathways enriched and p-values, q-value and NES can be found in supplementary table 3.V on the accompanying CD.

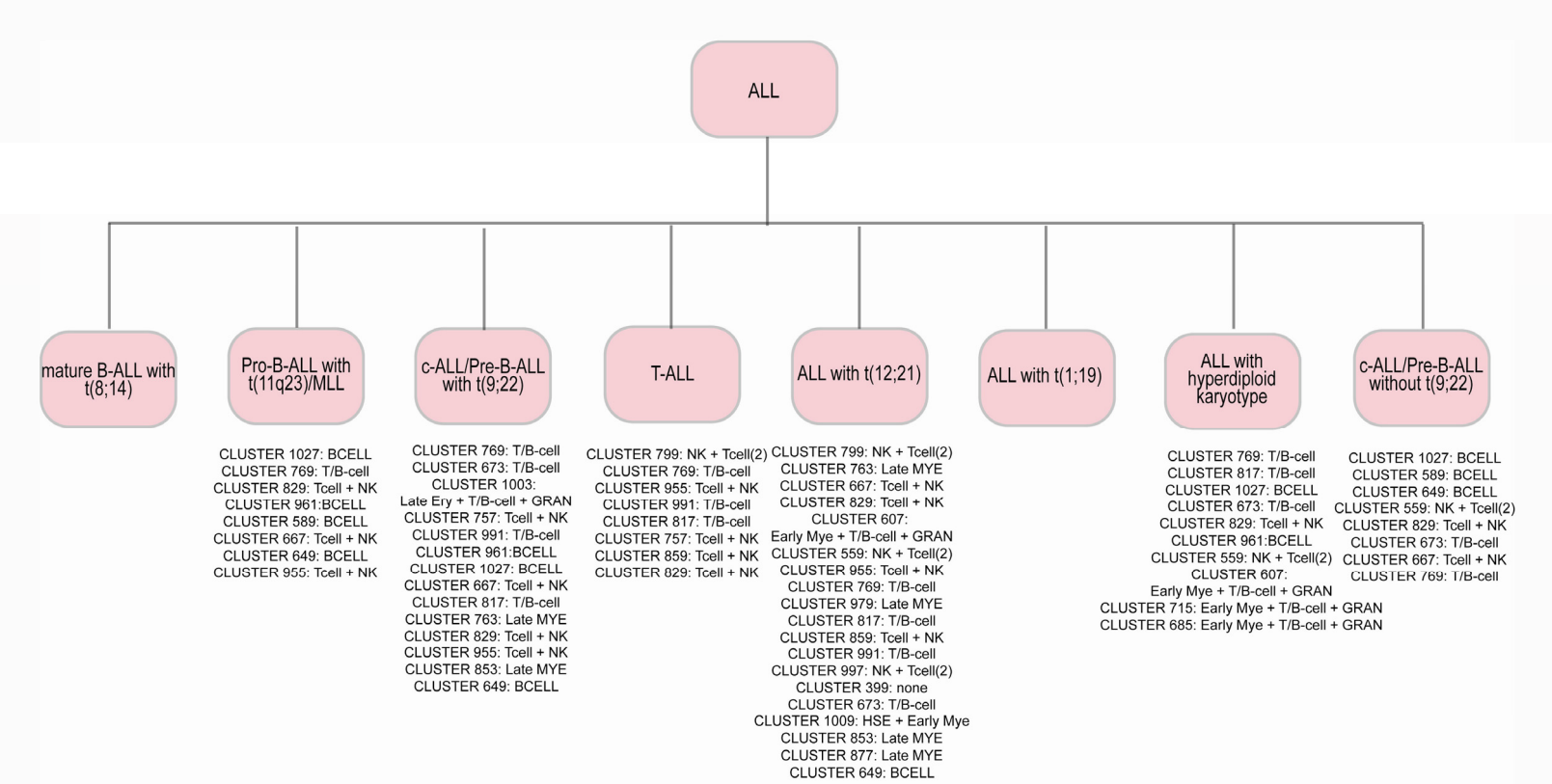

Figure 3.22: The haematopoietic gene modules enriched in the subsets of ALL found in the MILE Study for the TRIB2 signature. GSEA was carried out for the TRIB2 signature in all the leukaemic subtypes of the MILE study. GSEA was run using the haematopoietic gene modules, 80 modules of strongly co-expressed genes identified by Novershtern at al. in the haematopoietic cell lineages (Novershtern et al., 2011). Each module of genes was classified based on which cellular compartment the module was expressed in within the haematopoietic cell lineage. Enrichment for individual gene modules was determined for the TRIB2 signature for each of the leukaemic subtypes of the MILE study. Enriched gene module type can be seen listed in the above figure below the relevant leukaemia subtype ranked in order of negative enrichment score (NES). Detailed tables with names of individual pathways enriched and p-values, q-value and NES can be found in supplementary table 3.VI on the accompanying CD.

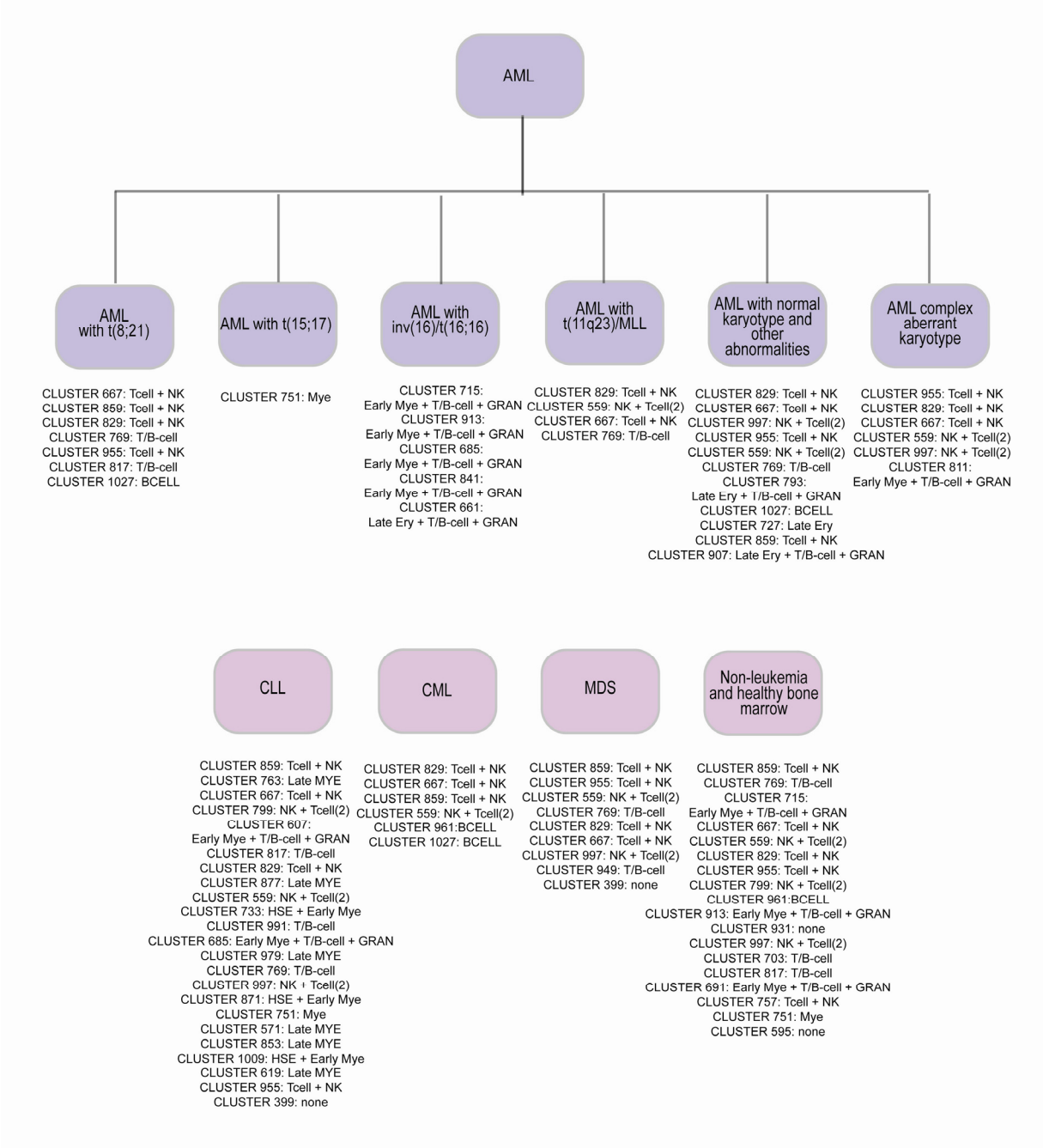

Figure 3.23: The haematopoietic gene modules enriched in AML subsets, CLL, CML, MDS and Non-leukaemic control group of the MILE Study for the TRIB2 signature. GSEA was carried out for the TRIB2 signature in all the leukaemic subtypes of the MILE study. GSEA was run using the haematopoietic gene modules, 80 modules of strongly co-expressed genes identified by Novershtern at al. in the haematopoietic cell lineages (Novershtern et al., 2011). Each module of genes was classified based on which cellular compartment the module was expressed in within the haematopoietic cell lineage. Enrichment for individual gene modules was determined for the TRIB2 signature for each of the leukaemic subtypes of the MILE study. Enriched gene module type can be seen listed in the above figure below the relevant leukaemia subtype ranked in order of negative enrichment score (NES). Detailed tables with names of individual pathways enriched and p-values, q-value and NES can be found in supplementary table 3.VI on the accompanying CD.

In contrast to the TRIB1 signature the TRIB2 signature identified enrichment for many gene clusters that are highly expressed in T cells and NK cells (Tcell + NK), in T and B cells (T/B cell) or in B cells only (BCELL) (figures 3.22 and 3.23). Enrichment for gene modules was found for the TRIB2 signature in all of the subtypes of leukaemia excluding mature B-ALL with t(8;14) and ALL with t(1;19). Other modules were also found to be enriched for the TRIB2 signature in some of the leukaemic subtypes. These include modules of genes highly expressed in late myeloid cells (Late MYE) which are enriched for the signature in, for example, the CLL patient samples. Also a number of modules of genes highly expressed across early myeloid, the T and B cells and the granulocytes (Early Mye + T/B-cell +GRAN) were found to be enriched in the AML with inv(16)/t(16;16) patient samples with the TRIB2 signature.

Analysis of the TRIB2 signature in the T-ALL patient samples revealed a large number of T cell and lymphoid compartment associated modules such as the T/B cell module (cluster 765) indicating that the TRIB2 signature is associated with lymphoid expressing genes in this leukaemic subtype (figure 3.22) (this data was published in the British Journal of Haematology, 2012 (see appendix A)). The TRIB2 signature is also associated with T cell modules in AML with normal karyotype and other abnormalities (figure 3.13). High TRIB2 expression has been previously linked to a T cell signature in AML with normal karyotype (Wouters et al., 2007). In the MILE study we see the association of a T cell signature with TRIB2 expression in AML once again. In contrast the TRIB1 signature is associated with modules containing late myeloid compartment genes (figure 3.20 and 3.21) further highlighting the differences in these two signatures in the leukaemic cells. The differences in the gene signatures suggest that TRIB1 and TRIB2 are associated with very different

pathways in the leukaemic cell though they both induce leukaemia (Keeshan et al., 2006; Jin et al., 2007; Dedhia et al., 2010).

Both the TRIB1 and the TRIB2 signatures are also enriched for a small number of similar modules e.g. the Late Mye module in the CLL samples. Expression analyses of TRIB1 and TRIB2 in the haematopoietic cells (figures 3.1 and 3.2) showed that TRIB1 expression was highest in the myeloid cells while TRIB2 expression was highest in the lymphoid compartment while clustering of TRIB1 and TRIB1 and their gene neighbours in the cells of haematopoiesis distinctly associated the TRIB1 signature with the myeloid compartment, specifically the monocyte cells and the TRIB2 signature with the lymphoid compartment (figures 3.1 and 3.2). The GSEA analyses' using the haematopoietic modules reflects this by revealing that each signature shows enrichment for distinct sets of modules in the leukaemic subtypes of the MILE study (figures 3.20, 3.21, 3.22 and 3.23). While the gene modules that are enriched in the TRIB1 signature are primarily associated with the myeloid cells the gene modules enriched for the TRIB2 signature are principally associated with the cells of the lymphoid compartment (B, T and NK cells) (figures 3.20, 3.21, 3.22 and 3.23).

3.4 Connectivity mapping using the gene profiles of TRIB1, TRIB2 and TRIB3 expression in leukaemia

In Section 3.2 gene signatures for high TRIB1, TRIB2 and TRIB3 expression in AML, ALL, CLL, CML and MDS were generated by determining the nearest-neighbours for each of the Tribble genes. In order to further utilize the information obtained from these gene signatures connectivity mapping was performed using the top 50 nearest neighbours of TRIB1, TRIB2 and TRIB3 in each of these disease states. Connectivity mapping aims to generate a detailed map that links gene patterns produced by drug candidates and a variety of genetic manipulations with gene patterns associated with disease (Lamb et al., 2006). Analyses using the TRIB1 gene signatures as determined in section 3.2 in the ALL, AML, CML and MDS samples of the MILE study identified a number of small molecules that positively connect to the TRIB1 gene signatures in these leukaemic disease states. These include ouabain (ChemBankID: 3172974), digoxin (ChemBankID: 1454) and digitoxigenin (ChemBankID: 3189314), which were in the top 10 hits for the TRIB1 signature in ALL, AML, CML and MDS (table 3.I). Results displayed in these tables are the top 10 permutated results ranked by small molecule for the cmap analyses of the TRIB1 signature in ALL, AML and CLL. Cmap name is the name of the drug, mean is the arithmetic mean of the connectivity scores for all instances with the drug, n is the number of those instances, enrichment is the measure enrichment of those instances in the order list of all instances (the closer to 1 the better), p is the permutation p-value for the enrichment score (significant is indicated by a value of less than 0.05), specificity is an estimate of the uniqueness of the connectivity between a set of instances and a signature of interest based (closer to 0 the better)and the non-null percentage, a measure of the support for the connection between a set of instances

and a signature of interest based upon the behaviour of the individual instances in that set. The threshold for the non-null percentage is 50%. The rows in the table are ordered in ascending order of p-value then ascending order of (absolute) enrichment.

These three small molecules are cardiac glycosides. They function by binding to and inhibiting the ubiquitous trans-membrane protein Na+, K+-ATPase. By inhibiting this trans-membrane pump they can increase the force of contraction of heart muscle and are often used in the treatment of heart failure (Balzan et al., 2000; Fürstenwerth, 2010). Cardiac glycosides have also been shown to regulate endocytosis and to relax cellular attachment (Contreras et al., 2004; Feldmann et al., 2007).

Vorinostat also connects with the TRIB1 signature in AML (table 3.I). Interestingly it has been recently reported that TRIB1 along with a number of other genes are induced by C/EBPα in leukaemic cells and that HDACI can positively induce this signature (Liss et al., 2013). Trichostatin A connects with the TRIB1 signature in ALL (table 3.I). Histone deactylases (HDACs) are proteins that regulate gene expression by deacetylation of the DNA leading to highly chromatin and transcriptional repression (Hoshino and Matsubara, 2010; Sharma et al., 2013). HDACs have also been shown to form complexes with E2F transcription factors and by doing so repress activation of E2F target genes (Chen et al., 2012; Emori et al., 2012; Sharma et al., 2013).

ALL							
rank	cmap name	mean	n	enrichment	p	specificity	percent non-null
1	ouabain	0.928	4	0.996	<0.00001	0	100
2	proscillaridin	0.899	3	0.995	<0.00001	0.0099	100
3	lanatoside C	0.89	6	0.99	<0.00001	0.0046	100
4	digoxin	0.9	4	0.989	<0.00001	0	100
5	digitoxigenin	0.924	4	0.988	<0.00001	0	100
6	helveticoside	0.883	6	0.986	<0.00001	0	100
7	anisomycin	0.687	4	0.957	<0.00001	0.0361	100
8	digoxigenin	0.835	5	0.957	<0.00001	0.0044	100
9	thioridazine	0.606	20	0.76	<0.00001	0.0091	90
10	trichostatin A	0.371	182	0.445	<0.00001	0.4123	71
AML							
rank	cmap name	mean	n	enrichment	p	specificity	percent non-null
1	digoxin	0.72	4	0.96	<0.00001	0.0047	100
2	digitoxigenin	0.676	4	0.957	<0.00001	0.0095	100
3	ouabain	0.697	4	0.946	<0.00001	0.0152	100
4	anisomycin	0.667	4	0.942	<0.00001	0.0412	100
5	lanatoside C	0.653	6	0.924	<0.00001	0.0092	100
6	helveticoside	0.729	6	0.915	<0.00001	0.017	100
7	pyrvinium	0.624	6	0.911	<0.00001	0.0093	100
8	vorinostat	0.579	12	0.772	<0.00001	0.1407	100
9	thioridazine	0.526	20	0.638	<0.00001	0.0776	85
10	trifluoperazine	0.396	16	0.604	<0.00001	0.0914	75
MDS							
rank	cmap name	mean	n	enrichment	p	specificity	percent non-null
1	digitoxigenin	0.728	4	0.975	<0.00001	0.0095	100
2	ouabain	0.663	4	0.953	<0.00001	0.0101	100
3	helveticoside	0.705	6	0.951	<0.00001	0.0085	100
4	digoxin	0.66	4	0.951	<0.00001	0.0047	100
5	8-azaguanine	0.667	4	0.942	<0.00001	0.0059	100
6	lanatoside C	0.617	6	0.884	<0.00001	0.0229	100
7	digoxigenin	0.583	5	0.906	0.00004	0.0044	100
8	thioridazine	0.397	20	0.532	0.00004	0.2055	70
9	pimethixene	0.631	3	0.942	0.00024	0	100
10	pimozide	0.514	4	0.878	0.0003	0.0201	100
CLL							
rank	cmap name	mean	n	enrichment	p	specificity	percent non-null
1	8-azaguanine	0.769	4	0.958	<0.00001	0	100
2	vorinostat	-0.55	12	-0.75	<0.00001	0.062	83
3	trichostatin A	-0.385	182	-0.499	<0.00001	0.1683	63
4	tretinoin	0.33	22	0.497	0.00002	0.0171	63
5	verteporfin	0.683	3	0.941	0.00026	0.0143	100
6	bisacodyl	0.7	4	0.876	0.00034	0	100
7	thioguanosine	0.577	4	0.872	0.00036	0.0177	100
8	STOCK1N-35215	0.661	3	0.93	0.00062	0	100
9	digitoxigenin	0.592	4	0.816	0.00211	0.0476	100
10	methylbenzethonium chloride	0.443	6	0.69	0.00228	0.0209	83
CML							
rank	cmap name	mean	n	enrichment	p	specificity	percent non-null
1	menadione	1	2	1	<0.00001	0	100
2	digitoxigenin	0.831	4	0.986	<0.00001	0.0048	100
3	ouabain	0.775	4	0.967	<0.00001	0.0051	100
4	helveticoside	0.783	6	0.888	<0.00001	0.017	100
5	lanatoside C	0.79	6	0.884	<0.00001	0.0229	100
6	thioridazine	0.387	20	0.621	<0.00001	0.1142	75
7	estradiol	-0.304	37	-0.398	<0.00001	0	51
8	camptothecin	0.89	3	0.993	0.00002	0.0848	100
9	digoxigenin	0.759	5	0.909	0.00004	0.0044	100
10	bepridil	0.671	4	0.904	0.00008	0	100

Table 3.I: Top 10 cmap small molecules enriched for the TRIB1 gene pattern in ALL, AML, MDS, CLL and CML. Complete list of enrichment results as well as break down of results based on cmap name and cell line or by atc code can be found in supplementary tables 3.VII to 3.XI on the accompanying CD.. TRIB1 signatures

used to run these analyses can be found in supplementary table 3.XII on the accompanying CD.

Analyses of the TRIB2 gene signature was performed excluding CML as less than 50 genes were found to be differently expressed between the high and low TRIB2 samples . High TRIB2 expression was most closely associated with the ALL leukaemic disease state (figure 3.2). Vorinostat (ChemBankID: 468) and Trichostatin A (ChemBankID: 199) are both histone deacetylase inhibitors (HDACI) and are the top ranked small molecules that connect to the TRIB2 signature in ALL (table 3.II). Vorinostat is also one of the top 10 small molecules that connect with the TRIB2 gene signature in CLL (table 3.II). Halofantrine (ChemBankID: 2080909) is an antimalarial drug that negatively connects with the TRIB2 signature in the ALL disease state. The mode of action of antimalarial drugs is unclear, however there is growing evidence for their use as anti-cancer agents (Kimura et al., 2013). It is believed that antimalarial drugs can inhibit autophagy, a cellular process that is linked to both the promotion and suppression of cancer, and are therefore being investigated as novel drugs for the treatment of cancer (Janku et al., 2011; Amaravadi et al., 2011; Rosenfeldt and Ryan, 2011; Kimura et al., 2013). Another member of the Tribble family, TRIB3, has already been linked to autophagy of cancer cell. TRIB3 can induce autophagy in human non-small cell lung cancer cells. By doing so it attenuated the apoptotic cascade in the tumour cell and this increased the survival of the cancer cell (Li et al., 2013). The fact that Halofantrine negatively links to a TRIB2 signature allows us to hypothesis that TRIB2 may also play a role in autophagy in the cancer cell and that Halofantrine may inhibit autophagy by regulating TRIB2 expression.

LY-294002 positively connects with the TRIB2 signature in AML (table 3.II). LY-294002 is a phosphoinositide 3-kinase (PI3K) inhibitor that was also found to negatively connects with the TRIB3 signature both in the AML, CLL and CML disease states (appendix D table DI). Aberrant PI3K/AKT signalling has been implicated in many cancers and specifically observed in AML (Martelli et al., 2010).TRIB3 has been shown to inactivate AKT in the liver (Du et al., 2003) and both TRIB2 and TRIB3 can inactivate AKT in adipocytes (Naiki et al., 2007). This analysis makes a negative connection between the TRIB3 signature, and a positive connection between the TRIB2 signature, and this PI3K inhibitor.

Connectivity mapping has identified potential small molecules that may induce or block a TRIB1, TRIB2 or TRIB3 gene signature associated in ALL, AML, MDS, CML or CLL. However as none of these findings were followed up in vivo or in vitro the findings of this *in silico* analysis are interesting but have not been validated and so any conclusions drawn are of limited value until confirmed at the bench. Connectivity mapping is a hypothesis generating tool and while it has identified some interesting small molecules further research will be needed to assess the effects, if any, that these small molecules have on Tribble expression in vitro and in vivo.

ALL							
rank	cmap name	mean	n	enrichment	p	specificity	percent non-null
1	vorinostat	0.655	12	0.816	<0.00001	0.1055	91
2	trichostatin A	0.508	182	0.584	<0.00001	0.1564	78
3	scriptaid	0.712	3	0.976	0.00004	0	100
4	carcinine	0.65	4	0.904	0.00008	0	100
5	halofantrine	-0.659	3	-0.955	0.00022	0	100
6	Prestwick-691	-0.569	3	-0.93	0.00052	0.0132	100
7	flecainide	-0.391	6	-0.744	0.00054	0	83
8	SC-19220	-0.527	4	-0.866	0.00062	0	100
9	benzbromarone	-0.546	3	-0.901	0.00184	0.0063	100
10	Prestwick-642	-0.434	4	-0.819	0.00203	0.0276	100
AML							
rank	cmap name	mean	n	enrichment	p	specificity	percent non-null
1	LY-294002	0.429	61	0.296	<0.00001	0.349	67
2	adiphenine	-0.632	5	-0.859	0.00008	0.0403	100
3	sirolimus	0.38	44	0.311	0.00024	0.3072	61
4	apramycin	0.753	4	0.878	0.0003	0	100
5	biperiden	-0.588	5	-0.825	0.00042	0.0204	100
6	sulfadimethoxine	-0.519	5	-0.813	0.00054	0	100
7	nadolol	-0.59	4	-0.871	0.00056	0.0077	100
8	betahistine	0.715	4	0.855	0.00058	0.0073	100
9	Prestwick-692	-0.597	4	-0.858	0.0007	0.0068	100
10	meteneprost	-0.614	4	-0.857	0.0007	0	100
CLL							
rank	cmap name	mean	n	enrichment	p	specificity	percent non-null
1	digitoxigenin	-0.736	4	-0.945	<0.00001	0	100
2	trichostatin A	0.297	182	0.487	<0.00001	0.3412	55
3	anisomycin	-0.709	4	-0.924	0.00004	0.0339	100
4	helveticoside	-0.491	6	-0.806	0.00014	0.013	100
5	15-delta prostaglandin J2	-0.398	15	-0.538	0.00016	0.0752	73
6	H-7	0.643	4	0.88	0.00028	0.0833	100
7	camptothecin	0.645	3	0.933	0.00052	0.1518	100
8	ouabain	-0.712	4	-0.849	0.00095	0.0351	100
9	lycorine	-0.528	5	-0.756	0.00156	0.08	80
10	0173570-0000	0.568	6	0.702	0.00183	0.0229	83
MDS							
rank	cmap name	mean	n	enrichment	p	specificity	percent non-null
1	monensin	-0.448	6	-0.782	0.00018	0	83
2	clemastine	-0.738	3	-0.942	0.00028	0	100
3	terazosin	-0.578	4	-0.889	0.00034	0	100
4	ethotoin	0.568	6	0.749	0.00068	0.0083	100
5	apramycin	0.675	4	0.836	0.00111	0	100
6	heptaminol	-0.425	5	-0.76	0.00148	0.0137	80
7	dicycloverine	-0.352	5	-0.753	0.0017	0.0076	60
8	nadolol	-0.537	4	-0.826	0.00177	0.0461	100
9	alprostadil	-0.49	7	-0.654	0.00178	0.016	85
10	practolol	0.613	4	0.816	0.00207	0	100

Table 3.II: Top 10 cmap small molecules enriched for the TRIB2 gene pattern in ALL, AML, MDS and CLL. Complete list of enrichment results as well as break down of results based on cmap name and cell line or by atc code can be found in supplementary tables 3.XIII to 3.XVI on the accompanying CD. TRIB2 signatures used to run these analyses can be found in supplementary table 3.XVII on the accompanying CD.

Discussion

Both Trib1 and Trib2, but not Trib3, are leukaemia causing genes primarily associated with the development of murine AML (Keeshan et al., 2006; Jin et al., 2007; Dedhia et al., 2010). Our analyses of TRIB1, TRIB2 and TRIB3 expression in the leukaemic subtypes of the MILE study (Haferlach et al., 2010) revealed that neither TRIB1 nor TRIB2 expression was significantly higher in the AML subtypes of the MILE study when compared to the control group. Furthermore, the TRIB1 signature did not cluster together in a specific leukaemic subtype for AML. Closer analyses of TRIB2 expression however did reveal that a subset of patient samples with AML with normal karyotype and other abnormalities showed increased TRIB2 expression compared to the normal bone barrow samples. Clustering of the TRIB2 signature in the AML samples also associated TRIB2 with AML with normal karyotype and other abnormalities and AML with complex aberrant karyotype. Elevated TRIB2 expression was also found in a subset of CLL patent samples when compared to the control group. Elevated TRIB2 expression has been linked to poor prognosis in CLL (Johansson et al., 2010). Elevated TRIB2 expression supports the hypothesis that elevated TRIB2 expression may also play a role in the pathogenesis of CLL in a subset of patients, as well as in AML.

Analyses of TRIB1, TRIB2 and TRIB3 expression in the ALL leukaemic subtypes showed that both TRIB1 and TRIB3 expression is significantly lower than expression in the control group. TRIB2 expression, however, proved to be significantly higher in the T-ALL, ALL with t(1;19) and mature B-ALL with t(8;14) leukaemic patient samples . Clustering of the TRIB2 signature also revealed that high expression of TRIB2 and its gene neighbours cluster together in the T-ALL and

in the ALL with t(1;19) patient samples strongly linking TRIB2 expression to these leukaemic subtypes. TRIB2 was identified as a Notch1 targeted gene in a T-ALL cell line (Keeshan et al., 2006) and in 2012 we published an analysis of paediatric T-ALL patients showing that TRIB2 expression is elevated in samples with NOTCH1 or F-box/WD Repeat-Containing Protein 7 (FBXW7) mutations compared to wild type patients samples (see appendix A for paper) (Hannon et al., 2012). Here we find elevated TRIB2 expression in T-ALL patient samples when compared to control group samples. TRIB2 expression was also found to be highest in the ALL t(1;19) patient samples. This data was included in the paper "Elevated TRIB2 with NOTCH1 activation in paediatric/adult T-ALL" published in 2012 (see appendix A). The t(1;19) translocation results in the fusion of the TCF3-PBX1 proteins (Hunger et al., 1991). TCF3 has been shown to act in a parallel pathway to Notch1 signalling in T cell development and both TCF3 and Notch1 regulate Hairy and Enhancer of Split-1 (Hes1) expression, a Notch 1 target, in a similar manner (Ikawa et al., 2006). Pre-B-cell Leukaemia Homeobox 1 (PBX1) has been shown to interact with the Hox family of proteins and form heterodimers (Shen et al., 1997; Wu et al., 2006), as well as with Meis1 (Shen et al., 1999). Meis1 and the Hox family of proteins have been shown to be involved in leukaemogenesis, specifically in AML (Eklund, 2011). Both TRIB1 and TRIB2 have been shown to cooperate with HoxA9 in order to induce AML (Jin et al., 2007; Keeshan et al., 2008).

While no subset of patient samples with increased TRIB1 expression was found in both the AML with normal karyotype and other abnormalities samples and in the CLL samples, a role for increased TRIB1 expression in myeloid leukaemia cannot be ruled out as a patient with increase TRIB1 expression has already been described (Röthlisberger et al., 2007). The limitations of microarray data may be the reason for

this discrepancy. The changes in gene expression patterns in microarray studies when comparing two different samples are a manifestation of all the cell types present in that sample. In the case of the MILE study the control sample were bone marrow samples with no selection for the stem cell carried out. Analyses of TRIB1 expression in the cells of the haematopoietic system (Novershtern et al., 2011) revealed that TRIB1 expression is highest in the myeloid cells, but is also high in the HSC, GMP and CMP cells. High TRIB1 expression in the progenitor cells of the haematopoietic system could mean that comparing expression of TRIB1 between the leukaemic cells and the control group (healthy bone marrow samples as well as samples from non-leukaemia conditions such as megaloblastic anaemia, haemolysis, iron deficiency, or idiopathic thrombocytopenic purpura) may mask increased TRIB1 expression in the leukaemic cells. Comparison between the mutated leukaemic cell and a haematopoietic progenitor where mutations that give rise to leukaemia often occur such as the CMP may be necessary to reveal perturbed TRIB1 expression in the disease state.

TRIB3 expression was found to be significantly higher in the AML with complex and aberrant karyotype samples compared to the control group samples. However clustering of TRIB3 and its gene neighbours in the AML samples do not cluster within these AML with complex and aberrant karyotype samples. In contrast to TRIB1 and TRIB2, TRIB3 is unable to induce AML in retroviral studies (Dedhia et al., 2010). Specifically this incapacity to induce leukaemia was associated with TRIB3's inability to degrade C/EBPα (Dedhia et al., 2010). Though elevated TRIB3 expression has been implicated in malignancies including colorectal cancer and breast cancer (Miyoshi et al., 2009; Wennemers et al., 2011a, 2011b) TRIB3 does not seem to have the same oncogenic effect of TRIB1 and TRIB2 in the

hematopoietic system. Elevated TRIB3 expression may result from oncogenic activity in these malignancies as increased TRIB3 expression occurs in stress and starvation conditions in tumours and may aid the ability of malignant cells to grow in the nutrient deficient and hypoxic environment typically found inside solid tumours (Bowers et al., 2003; Schwarzer et al., 2006).

Overall analyses of TRIB1, TRIB2 and TRIB3 expression in the cells of haematopoiesis revealed that while TRIB1 expression is highest in the myeloid compartment TRIB2 expression is highest in the lymphoid compartment. Specifically TRIB1 expression is highest in the monocytic cells and high expression of TRIB1 and its gene neighbours cluster together in this cellular compartment. TRIB2 expression is highest in T cells followed by the B cells and the NKA cells. High expression of TRIB2 and its gene neighbours cluster together in these three cellular compartments. TRIB3 expression is not as variable across the cellular compartments of haematopoiesis, though it is highest in the granulocytic cells. Clustering of TRIB3 and its gene neighbours did not reveal high TRIB3 expression clustering in any specific cellular compartment.

More detailed analyses of TRIB1, TRIB2 and TRIB3 expression in the subpopulations of haematopoietic cells revealed that both TRIB1 and TRIB2 expression vary markedly between the differentiation stages of haematopoietic cells. TRIB1 and TRIB2 expression significantly varies between the developing and mature T, B, NK, erythroid, dendritic and myeloid cells, potentially revealing a role for the regulated expression of TRIB1 and TRIB2 in haematopoietic cell differentiation.

Both TRIB1 and TRIB2 have been implicated in haematopoiesis. Increased TRIB2 expression has been shown to affect myelopoesis but not lymphopoiesis. In Trib2

chimeric mice enforced Trib2 over-expression had no effect on lymphoid cell development. However enforced Trib2 over-expression did inhibit the differentiation of granulocytic cells and promote the differentiation of monocytic cells. An increase in dendritic and macrophage cells was also observed in these mice (Keeshan et al., 2006). Trib1-deficient mice lack tissue-resident M2-like macrophages (associated with responses to anti-inflammatory reactions and tumour progression) and eosinophils due to aberrant C/EBPα expression (Satoh et al., 2013). The Tribble genes have also been associated with the control of adipocyte differentiation (Naiki et al., 2007).

Of interest is the opposing pattern of expression of TRIB1 and TRIB2 in the developing B, NK and monocytes cells for example. This divergent pattern of TRIB1 and TRIB2 expression suggests that opposing levels of expression of TRIB1 and TRIB2 may be important for haematopoietic differentiation.

Over expression of Trib2 in murine bone marrow cells leads to an increase in monocyte and a decrease in granulocyte production, and expression of Trib2 in normal myelopblasts (cells that do not normally express TRIB2) results in a block in Granulocyte colony-stimulating factor (G-CSF) induced differentiation (Keeshan et al., 2006). These data suggest that in granulocytic development if specifically TRIB2 levels are elevated above normal this may lead to a block in granulocytic development resulting in excessive monocytes production at the expense of the granulocytes.

GSEA of the TRIB1 and TRIB2 signature using the modules of haematopoietic genes showed that the TRIB1 TRIB2signature is enriched for genes associated with the myeloid cells while the TRIB2 signature is enriched for genes associated with the

lymphoid compartment. In the case of TRIB2 these analyses revealed that expression of TRIB2 and its gene neighbours is highest in the lymphoid compartment and cluster together in the T-ALL and ALL with t(1;19) patient samples. Aberrant expression of TRIB2 in the progenitor cells of haematopoiesis may thus lead to the transformation of these cells into leukaemic cells that express genes associated with the lymphoid compartment (T cells, B cells and NKA cells).

The connectivity mapping analyses using the TRIB1, TRIB2 and TRIB3 signatures in the AML, ALL, CLL, CML and MDS patient samples of the MILE study identified a number of drug candidates. Identification of cardiac glycosides potentially identified a link between TRIB1 expression endocytosis and cellular attachment. The linkage of TRIB2 expression to both HDACIs and antimalarial drugs indicates that TRIB2 expression may be linked to the activity of HDAC and that it may play a role in autophagy in the cell. The connectivity mapping analyses also connected TRIB2 and 3 expression to LY-294002. While this inhibitor may reverse the TRIB3 signature it is positively linked to the TRIB2 signature, further highlighting the similar and yet contrasting roles that the Tribble genes can play in the cell.

Altogether these data identify TRIB1 as a gene associated with myeloid expression in both normal and leukaemic cell. Though TRIB1 expression could not be directly linked to any specific subtype of leukaemia, its expression was linked to the expression of late myeloid genes in leukaemia. TRIB2 is overwhelmingly associated with the lymphoid compartment and is linked by connectivity mapping to HDAC activity, autophagy and the PI3K/AKT pathway. Finally TRIB3 expression is not specifically associated with any of the cells of haematopoiesis and while its expression does not vary during haematopoietic differentiation, it is highly expressed

in a number of leukaemic subtypes compared to the control group samples. In conclusion, there is limited evidence that TRIB3 may play a role in the development of leukaemia.

Overall these analyses of the microarray data has lead to some interesting observations and suggested many interesting leads for the investigation of the role the Tribble genes play in haematopoiesis and leukaemogenesis. However the limitations of microarray data and *in silico* analysis cannot be ignored. Specifically it must be recognised that microarray data can only provide information about the genes that are present on the array. Therefore we are potentially lacking information on the expression of any gene not included in the array. Microarray data also lacks information on micro RNA expression and so the data in a microarray can only give a limited snapshot to an aspect of gene expression. Added to this is the fact that any changes in gene expression between the samples are a manifestation of all the cell types present in each sample. Another limitation of microarray analysis is the fact that it consists of numerous error-prone steps which may also affect the results of the analyses. These facts must be borne in mind while analysing and interpreting the microarray data and *in silico* analysis done using this data. For the *in silico* analysis, as well as the limitations of the data used for the analysis, recognition of the fact that a computer program cannot mimic or take in to account the complexity of the cell, different cell types and cellular environments must also be kept in mind while interpreting the data. While these tools are powerful the results obtained must always be conformed in cell lines and mouse models where biological complexity can be included in the analysis.

Chapter 4

Analyses of the TRIB1 and TRIB2 signatures in leukaemia and in the normal cells of haematopoiesis

This work was published in part in British Journal of Haematology 158(5) 626-34; September 2012 entitled "Elevated TRIB2 with NOTCH1 activation in paediatric/adult T-ALL" Hannon MM, Lohan F, Erbilgin Y et al. (Appendix A).

Introduction

The Tribbles genes are often referred to as signal modulators involved in the complex regulation and transduction of both intra- and extracellular signalling pathways in the cell (Hegedus et al., 2007). As signal modulators dysregulation of the Tribbles genes have been associated with a vast array of diseases including cancer, leukaemia, inflammatory disorders and diabetes mellitus (Kiss-Toth et al., 2004; Zhang et al., 2005; Keeshan et al., 2006; Hegedus et al., 2007; Jin et al., 2007; Puiffe et al., 2007; Deng et al., 2009; Zanella et al., 2010; Izrailit et al., 2013; Koh et al., 2013).As potentials mediator of cellular signalling pathways the Tribbles genes are thought to control these various pathways through interaction with various kinases, transcription factors and ubiquitin ligases.

Functionally the Tribbles genes have been linked to a number of cellular pathways. Each of the Tribbles genes have been shown to act as adaptors in the MAPK signalling pathway (Kiss-Toth et al., 2004). The Tribbles proteins play a role in promoting the degradation of C/EBP proteins in the cell via direct protein interaction thereby influencing a number of cell processes including differentiation (Rørth et al., 2000; Keeshan et al., 2006; Yamamoto et al., 2007; Yokoyama et al., 2010; Dedhia et al., 2010). TRIB2 and TRIB3 expression inhibits the phosphoryation and activation of AKT and both Tribbles proteins can interact with AKT (Du et al., 2003; Ding et al., 2008; Xie et al., 2012). Furthermore, TRIB1 and TRIB3 have also been found to inhibit inhibit NF-κB mediated transcription in the cell (Wu et al., 2003; Ostertag et al., 2010; Duggan et al., 2010).

As TRIB1 and TRIB2 are leukaemia causing genes we were interested in discovering the potential pathways by which these genes can induce

leukaemogenesis. There is evidence that ***Trib***1 cooperates with HoxA9 and Meis1 and ***Trib***2 cooperates with HoxA9 in the induction of myeloid leukaemia (Jin et al., 2007; Keeshan et al., 2008a). Additionally, ***Trib***2 is also a potential downstream effecter of Meis1 leukaemogenic activity (Argiropoulos et al., 2008). In the leukaemic cell both can degrade C/EBPα in a COP1 dependent manner (Keeshan et al., 2010; Yoshida et al., 2013) which results in disrupted myelopoiesis and leads to the development of myeloid leukaemia in mice. ***Trib***1 has also been shown to link the MEF1/ERK pathway and the C/EBPα transcription factor in myeloid leukaemogenesis (Yokoyama et al., 2010).

Despite this knowledge the mechanism by which these genes contribute to leukaemogenesis has yet to be clarified. In this chapter we undertook a study using gene set enrichment analysis (GSEA) to identify potential pathways which TRIB1 and TRIB2 are associated with in both leukaemia and normal haematopoiesis. GSEA is a powerful tool that can be used widely to analyze and interpret microarray and RNA-Seq data. This analysis tool is capable of indentifying pathways and process from large scale datasets making the use of it a good starting point in identify potential pathways which TRIB1 and TRIB2 are associated with. There are some limitations to this approach however; GSEA sometimes has a low power as the recommended false discovery rate (FDR) is often 0.25 or below instead of the more robust 0.05 value normally used. Information is also lost in the GSEA analysis as relative gene rankings and not absolute measurements of gene expression is used in the analysis. Finally cases of GSEA identifying gene sets as statistically significant despite the fact that the genes of the gene set have no correlation with the phenotype or signature has been observed (Dinu et al., 2007). Despite these limitations GSEA is still a powerful tool suited to the discovery of pathways and genes associated with a

gene signature, in this case TRIB1 or TRIB2. Using the microarray data of the MILE (Haferlach et al., 2010) study and the haematopoietic cell data (Novershtern et al., 2011) the TRIB1 and TRIB2 signatures were analysed and a large number of potential pathways were identified. These pathways included the G-protein coupled receptor pathways, NF-κB pathways, Toll-like receptor (TLR) pathways and immune system signalling pathways for TRIB1, the T cell and T cell Co-Stimulation Pathways, TLR pathways, apoptosis pathways, B cell pathways and immune system signalling pathways for TRIB2.

As TRIB1 and TRIB2 expression are regulated in both the normal and leukaemic cell we also undertook a study to identify transcription factor targets that may be involved in this regulation. Once again GSEA analyses was utilised for the leukaemic data of the MILE Study (Haferlach et al., 2010) and the haematopoietic data (Novershtern et al., 2011) in order to identify transcription factor targets (TFTs) enriched for both the TRIB1 and the TRIB2 signatures in both these states. Transcription factor targets identified for TRIB1 included targets of the C/EBP family, Serum Response Factors (SRF), NF-κB and CREB and TRIB2 targets include ETS, Signal Transducers and Activators of Transcription (STAT), E2F, PAX and GATA.

Identifying the pathways and transcription factors involved in TRIB1 and TRIB2 regulation and activity in normal and leukaemic cells is an important part of determining the function of these two genes in normal haematopoiesis and their role in the development of leukaemia. Furthermore, identifying transcription factors that regulate the expression of these genes in haematopoietic or leukaemic cells may aid in the identification of potential therapeutic targets for further study.

Results

4.1 Profiling of the TRIB1 signature in the leukaemic subtypes of the MILE study

All leukaemic subtypes of the MILE study were analysed for enrichment of the canonical pathways for the TRIB1 signature (Supplementary Table 4.I found on the supplementary CD). As only the AML patient samples with normal karyotype contained a specific cluster of patient samples with a TRIB1 signature, analyses of the GSEA results are concentrated on these samples (figure 4.1). In the AML with normal karyotype and other abnormalities samples there is enrichment for multiple pathways including the complement pathways, TLR pathways, endocytic signalling pathways, haemostasis pathways, G-protein coupled receptor pathways, insulin signalling pathways, Wnt/β-catenin Signalling Pathway and NF-κB signalling pathways (Figure 4.1). TRIB1 has been previously associated with NF-κB signalling acting as a co-activator for RelA, a subunit of NF-κB, promoting the induction of proinflammatory cytokines in adipocytes (Ostertag et al., 2010).TRIB1 has also been found to negatively modulate C/EBPβ target genes in response to TLR signalling (Yamamoto et al., 2007) pathways.

Many of the above pathways are associated with immune system signalling and function including the G-protein coupled receptor pathways (Lattin et al., 2007), NF-κB pathways (Vallabhapurapu and Karin, 2009) and TLR pathways (Tapping, 2009). This suggests that TRIB1 may play an important role in the cellular signalling networks of the immune system and may act as a signal transducer in these pathways. A critical role for TRIB1 in the immune system is supported by the fact that Trib1-deficient mice lack tissue-resident M2-like macrophages, which are

associated with anti-inflammatory response and tumour progression due to aberrant C/EBPα expression (Satoh et al., 2013).

GSEA analyses of the TRIB1 signatures in the leukaemic subsets of the MILE study, this time using the chemical and molecular perturbation geneset file available from the Molecular Signatures Database (v3.0 MSigDB) was also carried out. This revealed a large number of genesets enriched in the various leukaemic subtypes of the MILE study. Many of the genesets enriched complement the results of the GSEA using the canonical pathway genesets (v3.0 MSigDB) for the TRIB1 signature (supplementary table 4.II found on the supplementary CD).

In the AML patient samples with normal karyotype and other abnormalities genesets were identified such as the those consisting of genes up regulated in primary fibroblast cells by expression of p50 (NFKB1) and p65 (RELA) (HINATA_NFKB_TARGETS_FIBROBLAST_UP) and those consisting of genes up-regulated in mammary epithelium cells by expression of constantly active β-catenin (CTNNB1) (KENNY_CTNNB1_TARGETS_UP) (supplementary table 4.II found on the supplementary CD). Aberrant Wnt/β-Catenin signalling pathway is associated with leukaemia and cancer and has been identified as a possible therapeutic target (Okuhashi et al., 2011; Memarian et al., 2012; Anastas and Moon, 2013). Other chemical and molecular signalling pathways enriched for the TRIB1 signature in the normal karyotype and other abnormalities leukaemic samples include genes up regulated in APL, a subtype of AML with t(15;17) inversion (APL) compared to normal promyeloblasts (CASORELLI_ACUTE_PROMYELOCYTIC_LEUKEMIA_UP). A large number of genesets whose expression is induced by stimulation of breast cancer or HELA

cells by Epidermal Growth Factor (EGF) a ligand of the Epidermal Growth Factor Receptor (EGFR) are also enriched. They include the AMIT_EGF_RESPONSE_120_HELA and NAGASHIMA_EGF_SIGNALING_UP genesets (supplementary table 4.II on the supplementary CD). The EGFR pathway has been implicated in tumourigenesis and the promotion of invasive, aggressive cancers (Feigin and Muthuswamy, 2009; Fry et al., 2009) and has not been previously linked to TRIB1 expression.

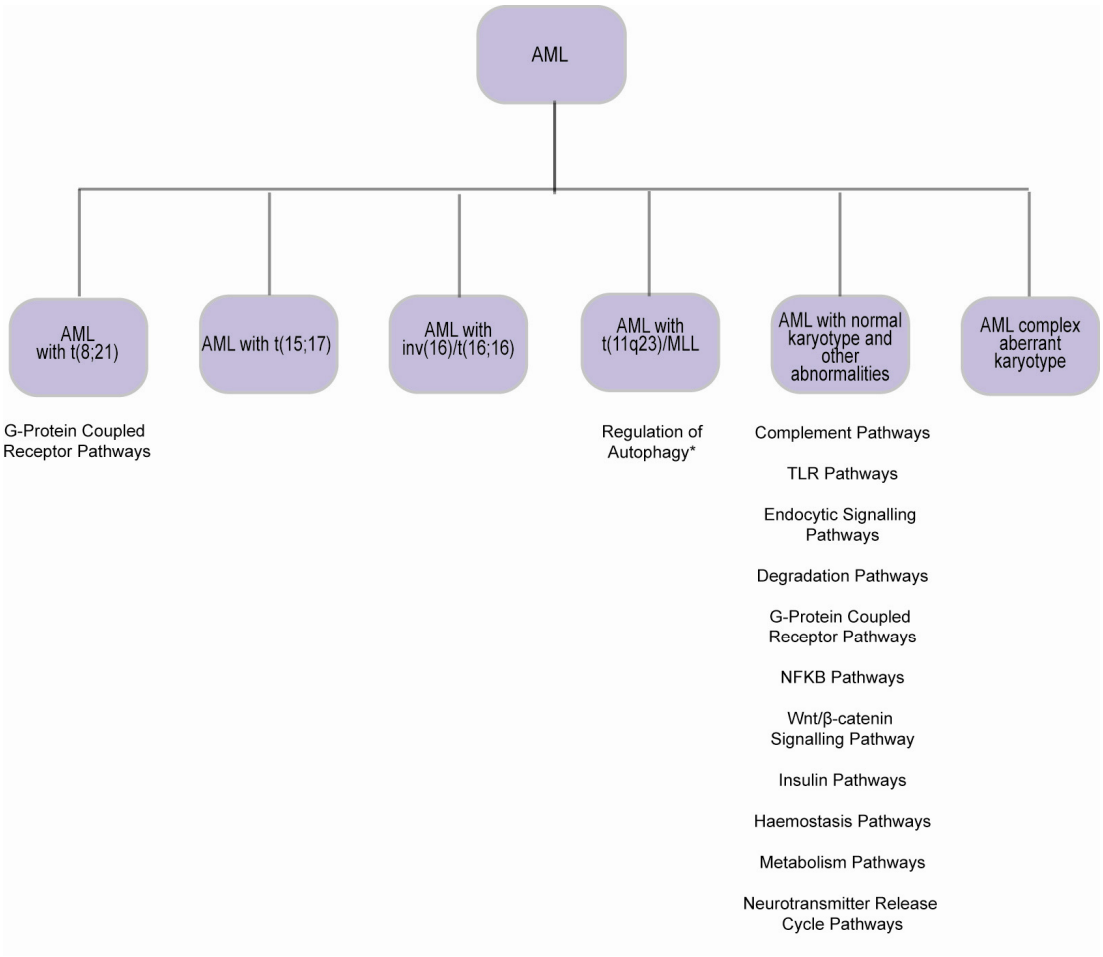

Figure 4.1: Canonical Pathways enriched in the subsets of AML in the MILE Study for the TRIB1 signature. GSEA analyses were carried out for the TRIB1 signature in the leukaemic subtypes of the MILE Study The pathways were then grouped based on type, pathway types with more than one hit can be seen listed in the above below the relevant leukaemia subtype, ranked in order of negative enrichment score (NES) (Detailed tables with names of individual pathways enriched and p-values, q-value and NES can be found in supplementary Table 4.I on the supplementary CD for all subsets of leukaemia.

As the TRIB1 signature in normal karyotype and other abnormalities AML samples is enriched for genes associated with APL we wished to investigate whether TRIB1 expression in non-APL AML samples gives an APL like signature. Therefore analyses of the TRIB1 signature in the non-APL AML samples found in the MILE study was carried out using genesets consisting of APL associated genes. These APL associated genesets are compiled of the top 200 genes up-regulated in the APL versus either the other AML samples in the Valk and Wouters AML datasets (Valk et al., 2004; Wouters et al., 2009) or the non-leukaemic samples of the Valk dataset (Valk et al., 2004). GSEA analyses demonstrated that the TRIB1 signature in the MILE study is enriched for APL associated genes indicating that AML with a high TRIB1 signature possesses a similar signature to APL (figure 4.2). As the TRIB1 signature is associated with an APL signature ATRA treatment may be of benefit to patients with a high TRIB1 signature as it is the standard treatment for APL (Tallman and Altman, 2009).

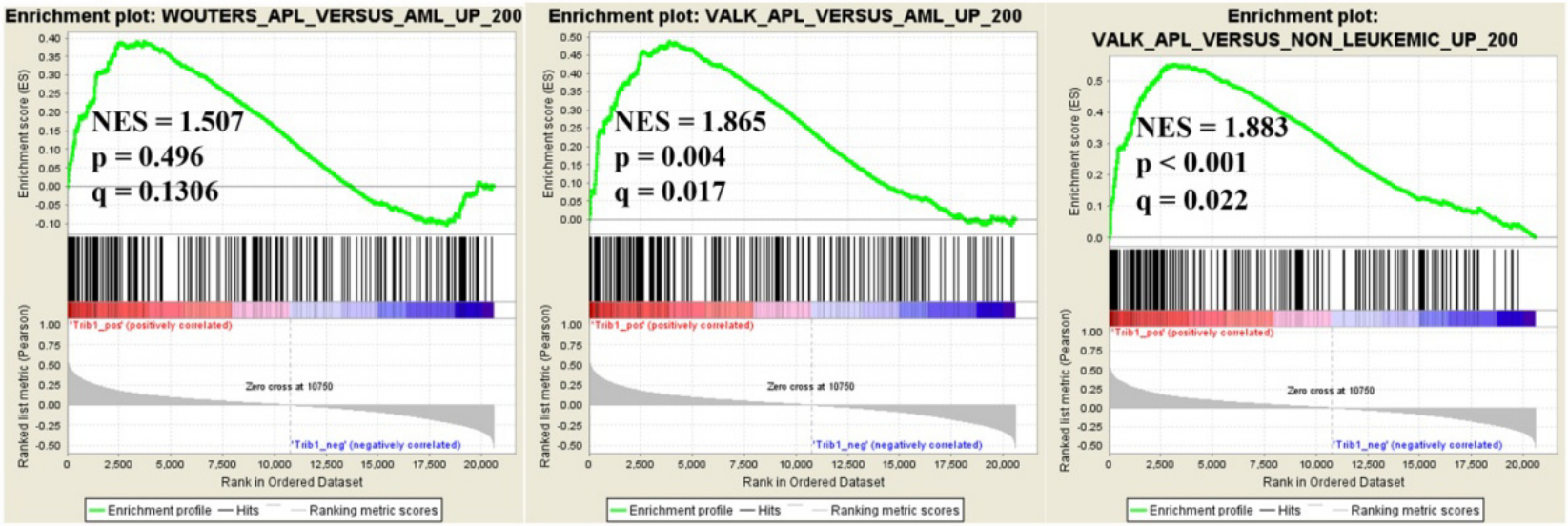

Figure 4.2: GSEA analyses of the TRIB1 signature in the AML samples of the MILE study excluding the APL samples using genesets consisting of the top 200 genes up-regulated in the APL samples of the Wouters (Wouters et al., 2009) and Valk (Valk et al., 2004) datasets compared to other AML samples and the top 200 genes up-regulated in the Valk dataset compared to non-leukaemic samples. The normalized enrichment scores (NES), p-value and q-value (FDR) are indicated on each plot; a p-value below 0.05 and a q-value below 0.25 indicates that the result is significant. When interpreting the above plots the top portion of the plot shows the running enrichment score (ES) for the gene set as the analysis walks down the ranked list, the middle portion of the plot shows where the members of the gene set appear in the ranked list of genes and the bottom portion shows the value of the ranking metric as you move down the list of ranked genes. The enrichment score is determined from where the top portion of the graph peaks.

4.2 Profiling of the TRIB1 signature in the cells and lineages of human haematopoiesis

GSEA was performed also for the TRIB1 signature in the cellular compartments and lineages of human haematopoiesis (Novershtern et al., 2011). These analyses were compared to the leukaemic cells of the MILE study. Most of the pathways enriched in the healthy cellular compartments and in the cell lineages were identical to the pathways enriched in the various leukaemic subtypes of the MILE study for the TRIB1 signature (figures 4.1 and 4.3). However some differences were found between the healthy cells and cell lineages and the leukaemic cells of the MILE study.

TRIB1 expression significantly increases as the monocytes mature (figure 3.11) and TRIB1 expression levels are highest in the monocytic compartment of the haematopoietic cells (figure 3.1). Due to this our analysis of the enriched canonical pathways concentrated on the monocytic lineage for the TRIB1 signature. Pathways enriched in the monocytic lineage for the TRIB1 signature include the haemostasis pathways, interleukin pathways, G-protein coupled receptor pathways, NF-κB pathways, TLR pathways, immune system signalling pathways, insulin pathways, T cell and T cell co-stimulation pathways, Nerve Growth Factor (NGF) pathways, cancer signalling pathways, apoptosis pathways, MAPK/ERK signalling pathways, B cell signalling pathways and endocytic signalling pathway (Figure 4.3). Transforming Growth Factor β (TGF-β) signalling pathways are enriched in the eosinophil, basophil and monocyte lineages for the TRIB1 signature (figure 4.3). The TGF-β growth factor family can act, depending on the type of tumour and the stage of tumour progression, as either a suppressor or promoter of the tumour growth and function (Akhurst and Hata, 2012). TGF-β plays an important role in immune

suppression; TGF-β1 null mice suffer from excessive inflammatory response and early death (Kulkarni et al., 1993). TGF-β has been shown to affect multiple cell lineages of haematopoiesis by promoting or opposing the differentiation, survival and proliferation of the cell lineages (Rubtsov and Rudensky, 2007). Here we see information that suggests that TRIB1 is associated with TGF-β signalling in the eosinophil, basophil and monocyte lineages (figure 4.3).

Analyses for the MILE study and the cellular compartments and lineages of haematopoiesis by GSEA using the chemical and molecular perturbation geneset file available for the Molecular Signatures Database (v3.0 MSigDB) revealed that many of the leukaemic subtypes and cell lineages of haematopoiesis are enriched with sets of genes that are targets of TGF-β signalling (supplementary tables 4.II and 4.IV on the supplementary CD) for the TRIB1 signature. The majority of these sets are of genes up-regulated in response to TGF-β1 stimulation. No TGF-β related genesets were enriched in the Granulocyte Lineage for the TRIB1 signature (supplementary table 4.IV on the supplementary CD). Together these data indicate that TRIB1 may be a target of TGF-β signalling in both normal and leukaemic cells. Just like the analyses for the MILE study, GSEA using the chemical and molecular perturbation geneset file available for the Molecular Signatures Database (v3.0 MSigDB) revealed a number of genesets enriched in the various leukaemic subtypes of the cells and cell lineages of haematopoiesis. These genesets include sets of genes up-regulated in response to EGF stimulation, in response to cytokine signalling, genes up-regulated by the inflammatory response, by AKT signalling and, finally, genes up-regulated in response to β-catenin (Supplementary Table 4.IV on the supplementary CD).

Figure 4.3: Canonical Pathways enriched in the myeloid cellular compartments and cell lineages of the haematopoietic cells for the TRIB1 signature. GSEA analyses were carried out for the TRIB1 signature in the cellular compartments and cell lineages of the haematopoietic cells. The pathways were then grouped based on type. Pathway types with more than one hit can be seen listed in the above, below the relevant leukaemia subtype ranked in order of negative enrichment score (NES) (Detailed tables with names of individual pathways enriched and p-values, q-value and NES can be found in the supplementary Table 4.III on the supplementary CD.

4.3 Profiling of the TRIB2 signature in the leukaemic subtypes of the MILE study

GSEA analyses of the enrichment of the canonical pathways in the TRIB2 signature of the ALL leukaemic subtypes revealed that pathways enriched in the ALL subtypes include the T cell and T cell Co-Stimulation Pathways, TLR pathways, apoptosis Pathways, B cell pathways and immune system signalling pathways (Figure 4.4). A number of these pathways which were also commonly found enriched in the MILE and haematopoietic cell and lineage groups for the TRIB1 signature (figures 4.1 and 4.3 and supplementary tables 4.I and 4.III on the supplementary CD). TRIB2 expression is highest in the lymphocyte compartment (figure 3.2) and together with these analysis, indicating that T cell and T cell Co-Stimulation Pathways are enriched for the TRIB2 signature, implies that TRIB2 may play an important role in lymphocyte signalling.

TRIB2 was identified as a Notch1 targeted gene in a T-ALL cell line (Keeshan et al., 2006) and the current analyses indicate that the Notch signalling pathway is enriched in the T-ALL samples (figure 4.4 (a and b)) (this data has been published in the British journal of haematology see appendix A). Along with the Wnt/β-catenin signalling pathways and the leukaemia and cancer associated pathways the Notch signalling pathway was only enriched in T-ALL of the ALL leukaemic subtypes of the MILE study (Figure 4.4).

The CREB pathway is involved in the regulation of cell survival, proliferation and the immune response. Over expression of CREB has been observed in both acute lymphoid or myeloid leukaemia (Cho et al., 2011). The ALL with t(1;19) patient samples, which show the highest TRIB2 expression of all the leukaemic subtypes

(figure 3.2) was discovered to be enriched for genes involved in the cAMP response element-binding protein (CREB) pathway (figure 4.4). This suggests a link between TRIB2 expression and the CREB pathway in ALL with t(1;19).

GSEA analyses for the TRIB2 signature in each of the AML subtypes of the MILE study revealed that, again, T cell and T cell Co-Stimulation Pathways, Apoptosis Pathways, B cell pathways, a number of interleukin pathways and immune system signalling pathways are, along with many other pathways, enriched across the numerous AML leukaemia subtypes (Figure 4.5). As in the ALL subtypes, the AML subtypes exhibit is strong enrichment for lymphocyte associated pathways for the TRIB2 signature in these leukaemias (figure 4.5).

A subset of normal karyotype AML with enhanced TRIB2 expression has been previously reported (Keeshan et al., 2006). This subset of AML was found to possess silenced C/EBPα expression and distinct expression of T cell associated genes such as CD7. Aberrant Notch signalling was also associated with this leukaemic subset (Wouters et al., 2007). As in the ALL subtypes, the AML subtypes exhibit a strong enrichment for lymphocyte associated pathways, including the AML with normal signature and other abnormalities samples, for the TRIB2 signature in these leukaemias (figure 4.5).

Pathways enriched only in the CLL subtype include the Wnt/β-Catenin signalling, Phospholipase C enzyme and Cell Adhesion Molecules (CAM) Signalling along with leukaemia and cancer associated signalling pathways (Figure 4.6).

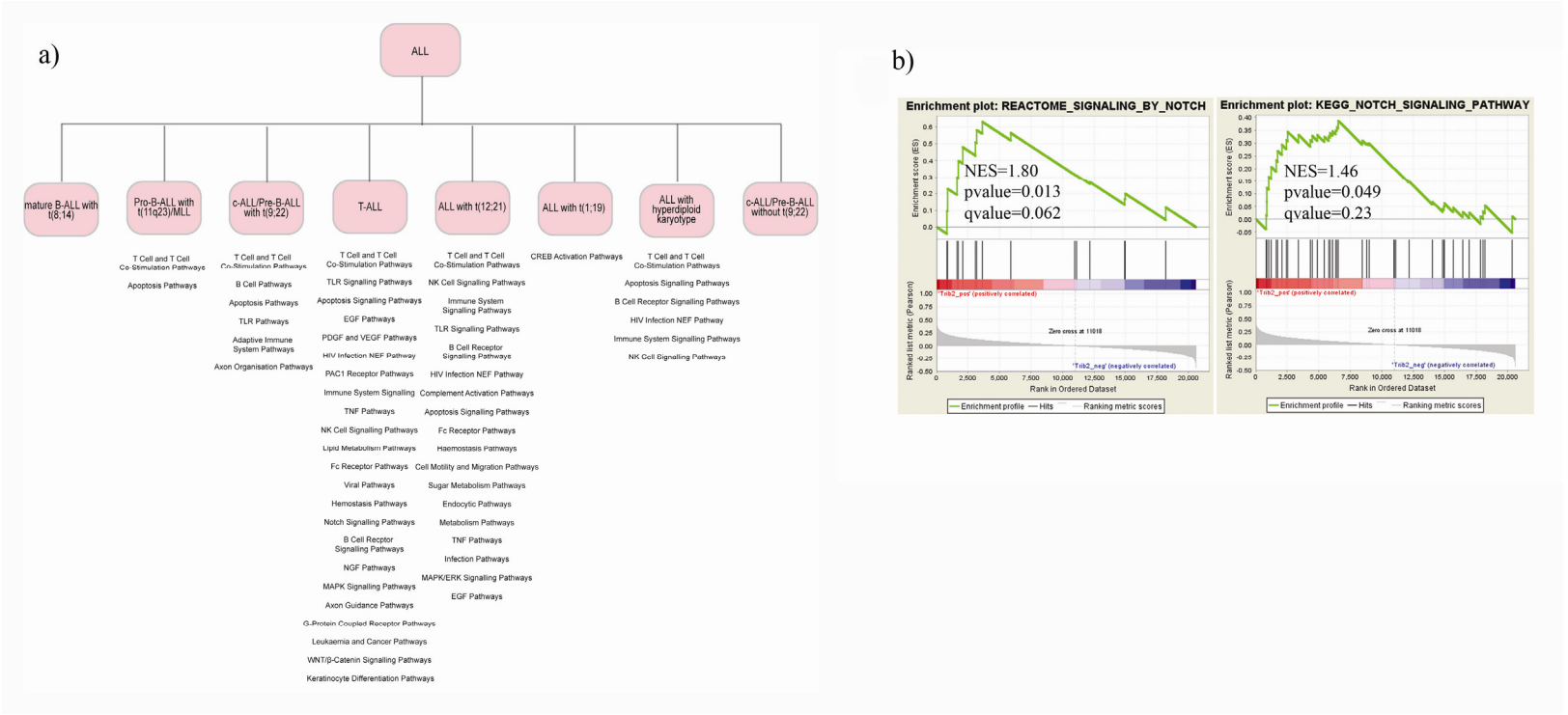

Figure 4.4: Canonical Pathways enriched in the ALL subsets of the MILE Study for the TRIB2 signature. GSEA analyses were carried out for the TRIB2 signature in the leukaemic subtypes of the MILE Study. The pathways were then grouped based on type. Pathway types with more than one hit can be seen listed in the above below the relevant leukaemia subtype ranked in order of negative enrichment score (NES). Detailed tables with names of individual pathways enriched and p-values, q-value and NES can be found in the supplementary Table 4.V on the supplementary CD. **b)** GSEA plots of the Notch Signalling Pathways enriched in the T-ALL subtype of leukaemia for the TRIB2 signature. Negative enrichment score (NES), the nominal p-values (NOM p-val) and false discovery rate (FDR) for each of the genesets are represented on the plots.

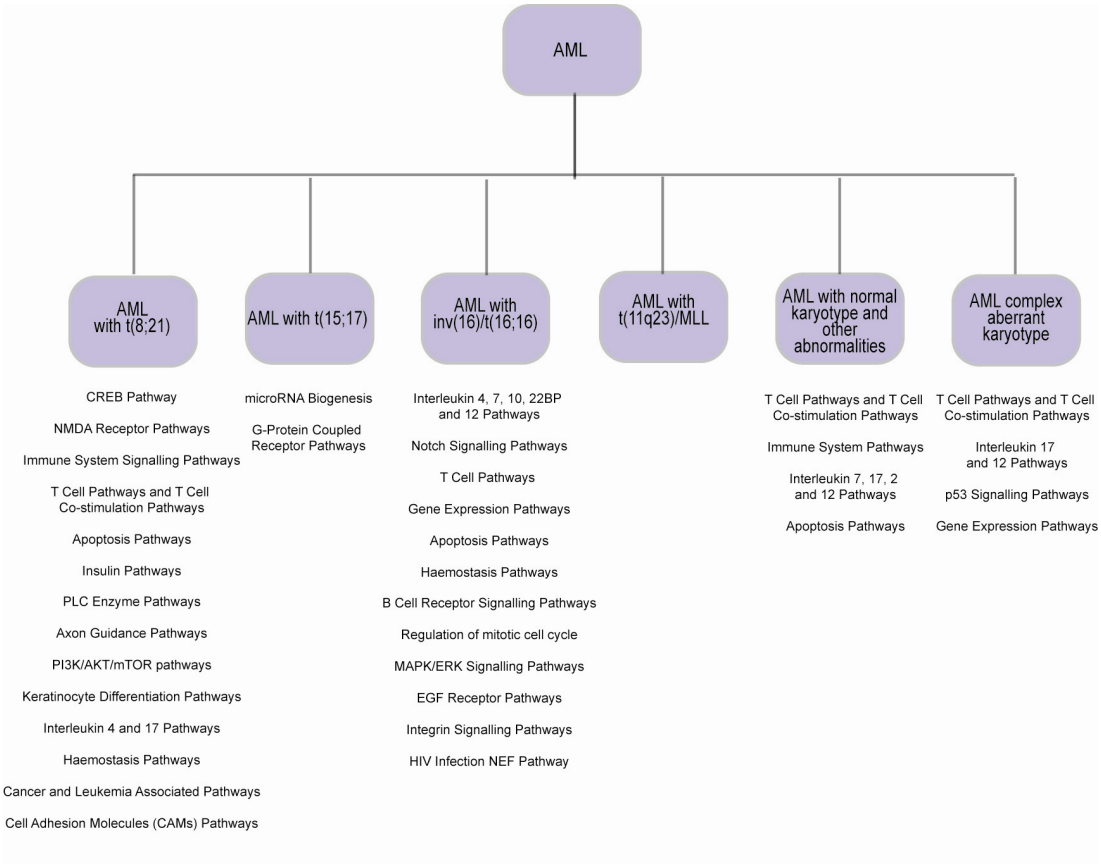

Figure 4.5: Canonical Pathways enriched in the AML subsets of the MILE Study for the TRIB2 signature. GSEA analyses were carried out for the TRIB2 signature in the leukaemic subtypes of the MILE Study. The pathways were then grouped based on type. Pathway types with more than one hit can be seen listed in the above below the relevant leukaemia subtype ranked in order of negative enrichment score (NES). Detailed tables with names of individual pathways enriched and p-values, q-value and NES can be found in the supplementary Table 4.V on the supplementary CD.

Figure 4.6: Canonical Pathways enriched in the subsets CLL, CML, MDS and non-leukaemic bone marrow samples of the MILE Study for the TRIB2 signature. GSEA analyses were carried out for the TRIB2 signature in the leukaemic subtypes of the MILE Study. The pathways were then grouped based on type. Pathway types with more than one hit can be seen listed in the above below the relevant leukaemia subtype ranked in order of negative enrichment score (NES). Detailed tables with names of individual pathways enriched and p-values, q-value and NES can be found in the supplementary Table 4.V on the supplementary CD.

TRIB2 may play a role in apoptosis signalling in the cell as the TRIB2 signature is highly enriched for apoptosis pathways in the ALL, AML, CLL, CML and MDS leukaemic subtypes (figures 4.4, 4.5 and 4.6). Both TRIB1 and TRIB2 have been reported to have pro-apoptotic affects in the cell (Lin et al., 2007; Gilby et al., 2010; Keeshan et al., 2010; Grandinetti et al., 2011).

Analyses for the MILE study by GSEA using the chemical and molecular perturbation geneset file available for the Molecular Signatures Database (v3.0 MSigDB) revealed that sets of genes up-regulated during apoptosis are enriched in the mature B-ALL with t(8;14) and ALL with t(12;21) subtypes of ALL, which both have significantly lower TRIB2 levels compared to the control group samples (figure 3.2), as well as in the CLL patient samples (Supplementary Table 4.VI found on the supplementary CD). (Genesets enriched include HOLLMANN_APOPTOSIS_VIA_CD40_UP and GALI_TP53_TARGETS_APOPTOTIC_UP).

TRIB2 expression is most strongly associated with AML with normal karyotype to date (Keeshan et al., 2006) and a subset of these patients in the MILE study demonstrated increased TRIB2 expression compared to the control group samples (figure 3.3). The pathways enriched for the TRIB2 signature in these patient samples include T cell pathways and T cell co-stimulation pathways and apoptosis pathways (figure 4.5) indicating that the TRIB2 signature is associated with T cell pathways and that AML with high TRIB2 expression may have a T cell signature. This analysis is supported by the finding that a subset of AML with high TRIB2 expression has a T cell signature as these samples had high expression of T cell genes such as CD7 and Notch1 (Wouters et al., 2007).

T cell and T cell Co-Stimulation Pathways are also enriched across the MILE study for the TRIB2 signature (figures 4.4, 4.5 and 4.6). When the MILE study was analysed using the chemical and molecular perturbation geneset file available for the Molecular Signatures Database (v3.0 MSigDB) genesets comprised of genes up-regulated at different points of the T cell differentiation stages were found to be enriched for the TRIB2 signature across the ALL and AML subtypes as well as in the CLL, CML, MDS and even the control group samples (Supplementary Table 4.VI). These include genes that are down-regulated at the early stages of progenitor T lymphocyte maturation compared to the later stages (LEE_EARLY_T_LYMPHOCYTE_DN) (Lee et al., 2004), which is enriched in, for example, the T-ALL samples for the TRIB2 signature, and NK lineage and T lymphocyte lineage associated genes (HADDAD_T_LYMPHOCYTE_AND_NK_PROGENITOR_UP) (Haddad et al., 2004) which are enriched in the AML with normal karyotype and other abnormalities samples.

4.4 Profiling of the TRIB2 signature in the cells and lineages of human haematopoiesis

GSEA analyses of the TRIB2 signatures in the individual cellular compartments of the haematopoietic system revealed that the HSC, GMP, Pre-B cells, NK cells, Mature B Cells, the Basophils and the Eosinophils were the only cells types that had significant enrichments for canonical pathways when analysed (Figure 4.7). Pathways involved in the regulation of gene expression and cell cycle regulation were found to be enriched in the Pre-B cells, the NK cells the Basophils and the Eosinophils (only gene expression pathways were enriched in the Eosinophils)

(Figure 4.4(a)). Other pathways that are enriched include interleukin pathways (NK cells, pre-B cells and Basophils), MAPK/ERK pathways (pre-B cells and Basophils), B cell signalling pathways (pre-B cells and Basophils) and various growth factor pathways (mature B cells and Basophils) (Figure 4.7).

Analysis of the lineages of the haematopoietic system for the TRIB2 signature found that only the T cell, B cell and Granulocytic lineages are enriched for various canonical pathways (figure 4.8). Like the MILE study B cell pathways, a number of interleukin pathways and immune system signalling pathways are enriched across the three different lineage types (figure 4.8).

The T cell and T cell Co-Stimulation Pathways, and Apoptosis Pathways were found to be enriched in the T cell and granulocytic lineages (figure 4.8). Uniquely in the T cell lineage the Her/ErbB pathway was enriched and in the B cell lineage the NF-κB pathway was found to be enriched (Figure 4.8). Unique to the granulocytic lineage is the enrichment of differentiation and cell migration pathways along with Rho signalling pathways. These pathways were not found to be enriched in the analyses of the MILE study for the TRIB1 signature (figures 4.4, 4.5 and 4.6). These analyses indicate that these pathways are unique to the TRIB2 signature in the normal cell lineage compared to the leukaemic cells.

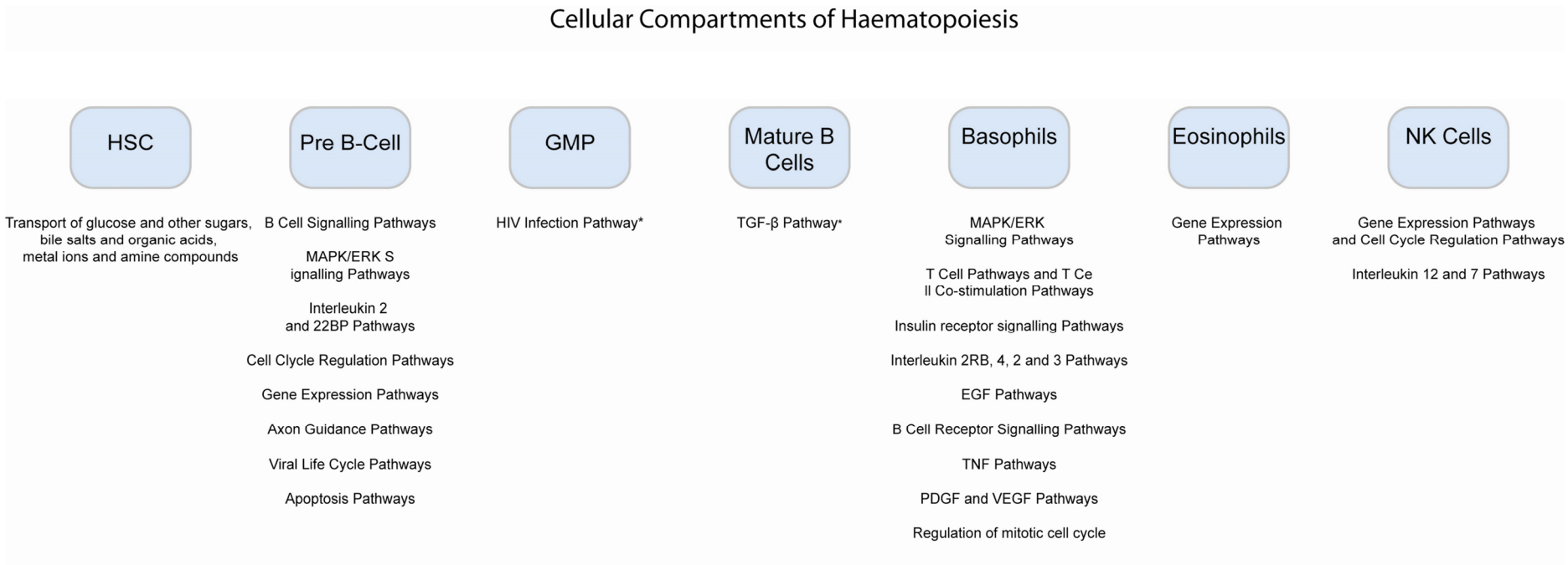

Figure 4.7: Canonical Pathways enriched in the cellular compartments for the TRIB2 signature. GSEA analyses were carried out for the TRIB2 signature in the cellular compartments and cell lineages of the haematopoietic cells. The pathways were then grouped based on type, pathway types with more than one hit can be seen listed in the above below the relevant leukaemia subtype ranked in order of negative enrichment score (NES). Detailed tables with names of individual pathways enriched and p-values, q-value and NES can be found in the supplementary Table 4.VII on the supplementary CD.

Figure 4.8: Canonical Pathways enriched in the cell lineages of the haematopoietic cells for the TRIB2 signature. GSEA analyses were carried out for the TRIB2 signature in the cellular compartments and cell lineages of the haematopoietic cells. The pathways were then grouped based on type, pathway types with more than one hit can be seen listed in the above below the relevant leukaemia subtype ranked in order of negative enrichment score (NES). Detailed tables with names of individual pathways enriched and p-values, q-value and NES can be found in the supplementary Table 4.VII on the supplementary CD.

Aberrant PI3K/AKT signalling has been implicated in many cancers but specifically in AML (Martelli et al., 2010). Analysis of the B cell and Granulocytic lineages for the TRIB2 signature reveal that the PI3K/AKT pathway is enriched in these lineages (figure 4.8). The TRIB1 signature is also enriched for this pathway in the Basophil, Eosinophil, Monocyte, Megakaryocyte and NK cell lineages(Figure 4.3).. However TRIB2 has been shown to inactivate AKT in the myeloid cells (Keeshan et al., 2010) and PI3K/AKT signalling pathway was found to be enriched in AML with t(8;21) for the TRIB2 signature (figure 4.5), an AML signature. This AML subtype has significantly lower TRIB2 expression than the control group (figure 3.2) indicating the TRIB2 is not active in this leukaemic subtype. It has been suggested that the AKT pathway is a potential therapeutic target in AML (Martelli et al., 2010) however these data indicate that it may not be beneficial in AML with abnormal TRIB2 expression.

4.5 Identification of Transcription Factor Targets (TFTs) associated with the TRIB1 signature in the leukaemic subtypes of the MILE study

Identifying transcription factors involved in TRIB1 and TRIB2 regulation and activity in normal and leukaemic cells is an important part of determining the function of these two genes in normal haematopoiesis and their role in the development of leukaemia. GSEA analyses for the TRIB1 signature in both the leukaemic subsets of the MILE study and in the human hematopoietic cells (Novershtern et al., 2011) resulted in the identification of potential transcription factor regulators of TRIB1 expression both in leukaemia. Since a number of patient

samples cluster with the TRIB1 signature in the normal karyotype and other abnormalities (figure 3.16) our analysis focused on these samples.

A large number of different transcription factor targets (TFTs) are enriched in the AML with normal Karyotype and other abnormalities when analysed using the TRIB1 signature (figure 4.9). Enriched TFTs include targets of the C/EBP family, SRF, NF-κB and CREB transcription factors (figure 4,5).

SRFs are transcription factors associated with cancer (Kim et al., 2009; Kwon et al., 2010) and insulin resistance (Jin et al., 2011). SRF is a member of the MADS family that mediates transcriptional activation in the cell in response to serum factors by binding to regions of the DNA known as Serum Response Elements (SRE) (Treisman, 1986, 1987; Norman et al., 1988; Shore and Sharrocks, 1995; Chai and Tarnawski, 2002; Miano, 2003). SRF are involved in various cellular processes such as the expression of tissue specific genes, cell proliferation, differentiation and apoptosis as well as inducing the expression of immediate early genes like c-fos and Early Growth Response Protein 1 (Egr-1) (Camoretti-Mercado et al., 2000; Bertolotto et al., 2000; Schratt et al., 2001; Zhang et al., 2001; Ding et al., 2001; Chai and Tarnawski, 2002)The TRIB1 signature is enriched with target genes of the SRF transcription factor in the AML with normal karyotype and other abnormalities patient samples (figure 4.10 (a)).

Complementing the above results and indicating that TRIB1 may be involved in regulating the cellular response to serum are the analysis showing that serum response chemical and molecular genesets are also enriched for the TRIB1 signature across all of the leukaemic subtypes of the MILE study excluding only mature B-ALL with t(8;14) (Supplementary Table 4.II found on the supplementary CD). To

support this analysis, an experiment was performed in the lab to test the gene expression levels of TRIB1 following serum stimulation in the U937 human AML cell line. These cells were chosen as they are a myeloid cell line that can undergo monocytic differentiation (ATCC CRL-1593.2) and control 293T fibroblast cells were also chosen as they are a non-leukaemic cell line (ATCC CRL-3216). U937 cells were serum deprived for 24 hours and then stimulated with 10% serum. mRNA taken at 16, 20 and 24 hours post stimulation (figure 4.10 (b)). TRIB1 expression increased and peaked at 16 hours and slowly dropped at 20 and 34 hours. This induction was not found in the 293T cell line indicating that the serum response may be specific to AML cells.

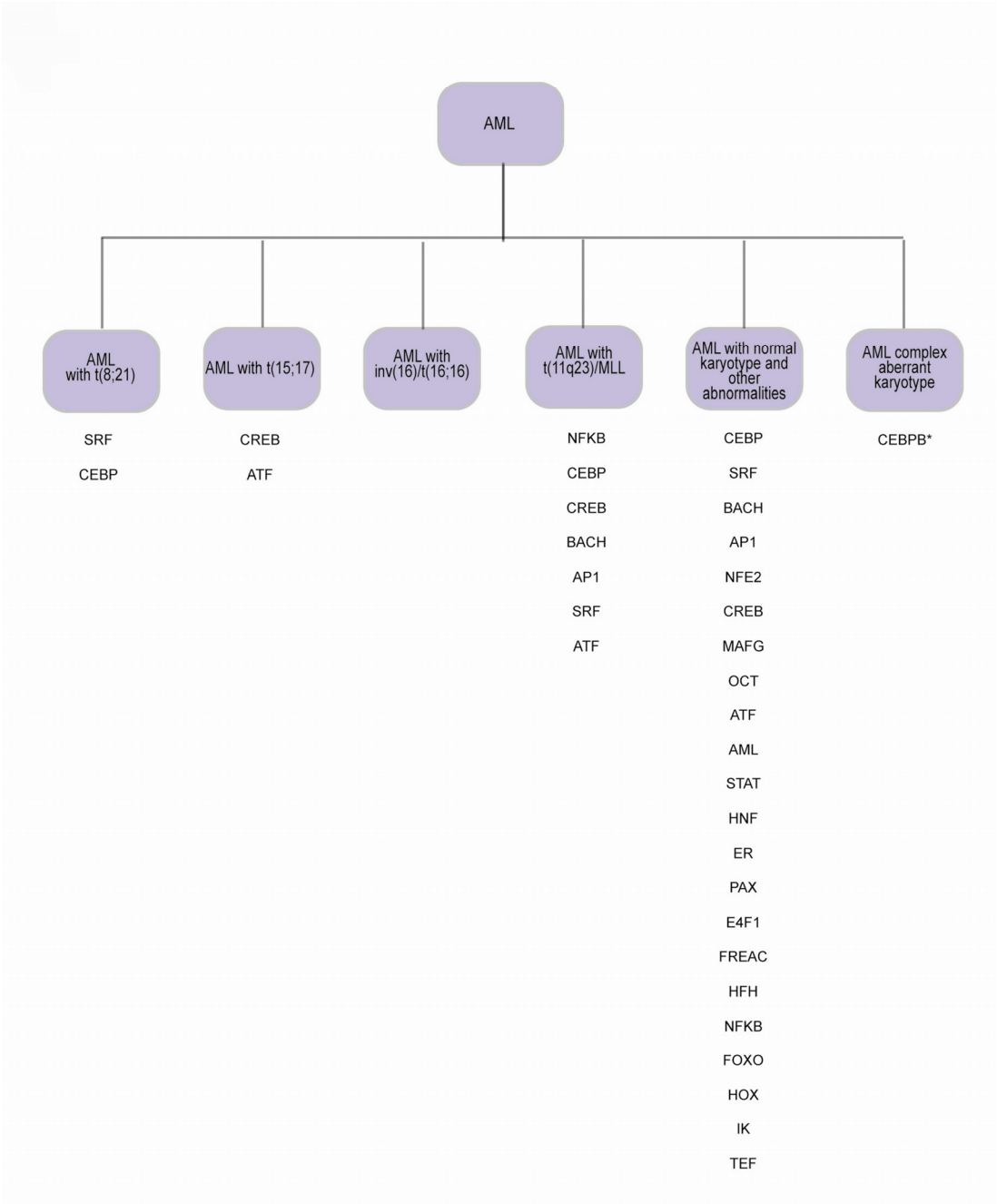

Figure 4.9: TFTs enriched in the AML subsets of the MILE Study for the TRIB1 signature. GSEA analyses were carried out for the TRIB1 signature in the leukaemic subtypes of the MILE. The TFTs were then grouped based on type, TFT types with more than one hit can be seen listed in the above below the relevant leukaemia subtype ranked in order of negative enrichment score (NES). Detailed tables with names of individual pathways enriched and p-values, q-value and NES can be found in the supplementary Table 4.IX on the supplementary CD.

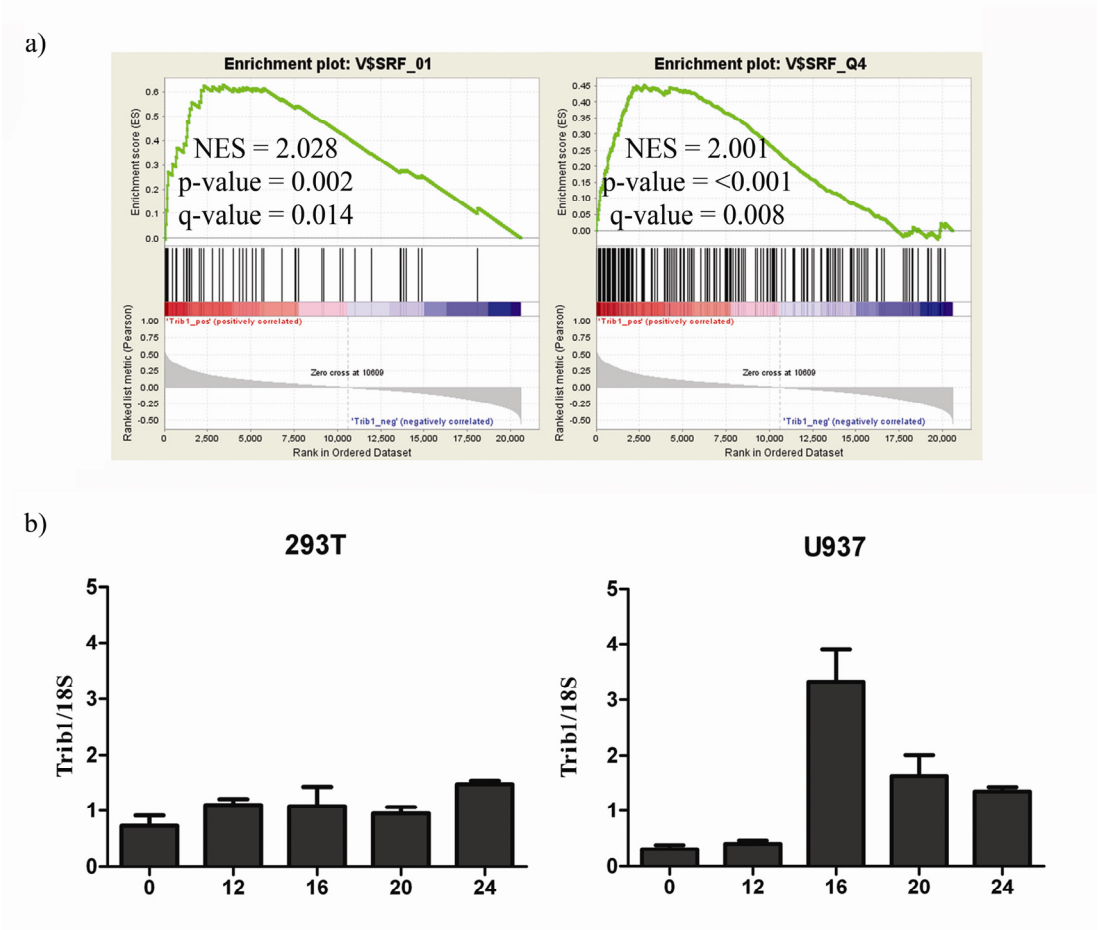

Figure 4.10: a) GSEA plots of the two most highly enriched SRF transcription factor target genesets and detailed table of the SRF transcription factor target genesets that are enriched in the AML with normal karyotype and other abnormalities leukaemic subtype for the TRIB1 signature. The negative enrichment score (NES), the nominal p-values (p-value) and false discovery rate (q-value) for each genesets is written on each plot. **b)** TRIB1 expression is induced in response to serum stimulation in U937 but not 293T cells. Cells were serum starved for 24 hours before 10% FBS was added to the media. TRIB1 mRNA levels were then measured at 0, 12, 16, 20 and 24 hours after addition of serum. Values are representative of experiments performed in duplicate. Error bars indicate +/- SD.

Other transcription factor targets enriched in the AML with normal karyotype and other abnormalities for the TRIB1 signature include NF-κB and CREB (figure 4.9). The NF-κB signalling pathway is associated with cancer development and progression (Karin, 2006) and are critical regulators of haematopoiesis (Denk et al., 2000). While CREB is also a critical transcription factor involved in haematopoiesis and leukaemogenesis (Cho et al., 2011).

Genesets of NF-κB targets from the chemical and molecular perturbation geneset file (v3.0 MSigDB) are enriched for the TRIB1 signature in the AML with normal karyotype and other abnormalities subtype of the MILE study (Supplementary Table 4.II found on the supplementary CD). Gene sets enriched include HINATA_NFKB_TARGETS_KERATINOCYTE_UP, a set of genes up-regulated in primary keratinocytes by expression of p50 (NFKB1) and p65 (RELA) both components of NF-κB (Hinata et al., 2003).

Enrichment of targets of the HOX family of transcription factors was found only in AML with normal karyotype and other abnormalities, patient samples for the TRIB1 signature is intriguing as Trib1 has been shown to cooperate with HOXA and MEIS1 in myeloid leukaemogenesis (Jin et al., 2007). Trib2 has also been shown to cooperates with a particular member of the HOX family, HOXA9, to accelerate AML in mice (Keeshan et al., 2008b). The modulation of the expression of members of the HOX gene family is involved in body patterning during development (Pearson et al., 2005) and is a key process in haematopoiesis; dysregulation of these genes leads to a block in myeloid differentiation and eventually leukaemia (Magli et al., 1997; Eklund, 2011). GSEA analyses using the chemical and molecular perturbation geneset file (v3.0 MSigDB) identified a number of genesets of genes both up and down regulated enriched for the TRIB1 signature in the normal karyotype subtype of

AML in the MILE study (Supplementary Table 4.II found on the supplementary CD). Gene sets include HESS_TARGETS_OF_HOXA9_AND_MEIS1_DN which is composed of genes down-regulated in hematopoietic precursor cells conditionally expressing HOXA9 and MEIS1 (Hess et al., 2006). Also the TAKEDA_TARGETS_OF_NUP98_HOXA9_FUSION_10D_DN geneset which is a collection of genes down-regulated in CD34+ hematopoietic cells by expression of NUP98-HOXA9 fusion of a retroviral vector at 10 days after transduction (Takeda et al., 2006). Other genesets from A. Takeda et al. of genes down-regulated in CD34+ hematopoietic cells by expression of NUP98-HOXA9 fusion of a retroviral vector at 6 hours, 3 days and 8 days are also enriched for the TRIB1 signature (Supplementary Table 4.II found on the supplementary CD).

The AML with normal karyotype and other abnormalities samples the geneset also enrich for genesets of targets positively regulated by the HOX genes. These include the TAKEDA_TARGETS_OF_NUP98_HOXA9_FUSION_16D_UP (Takeda et al., 2006) geneset. It consists of genes up-regulated in CD34+ hematopoietic cells by expression of NUP98-HOXA9 fusion of a retroviral vector at 16 days after transduction. Also enriched is the CHEN_HOXA5_TARGETS_6HR_UP (Chen et al., 2005) geneset which is made up of genes up-regulated 6 h after induction of HoxA5 expression in a breast cancer cell line.

These analyses suggest that the TRIB1 signature may cooperate or associate and may even posses a similar gene signature as the oncogenic Hox gene.

4.6 Identification of Transcription Factor Targets (TFTs) associated with the TRIB1 signature in the cellular compartments and the cellular lineages of the human haematopoietic system

GSEA analyses of each of the cellular lineages of the haematopoietic system using the TRIB1 signature established that only the T cell, monocyte, granulocyte and megakaryocyte lineages are enriched for various groups of TFTs (Figure 4.11. Indeed, TRIB1 expression is highest in the monocytes (figure 3.1) and significantly changes during monocytic differentiations (figure 3.11). TFTs enriched for the TRIB1 signature in the monocytic lineage include PU1, SRF, NFKB and CEBPB (figure 4.11).

PU.1 (SPI1) is a member of the ETS family and is the major ETS factor involved in haematopoiesis. It is particularly important in the regulation of gene expression during myeloid cell development (Sharrocks et al., 1997). PU.1 is a master regulator of transcription and plays a central role as a primary transcriptional determinant of hematopoietic cell fate (Burda et al., 2010). PU.1 has been shown to be a tumour suppressor in myeloid leukaemia, however an increase in the levels of PU.1 expression in early T cells leads to the development of T cell leukaemia (Kastner and Chan, 2008). TFTs of PU.1 are enriched for the TRIB1 signature during myeloid cell development (monocyte and megakaryocyte lineages (figure 4.11) indicating a potential role for PU.1 as a regulator of TRIB1 expression in these lineages. Lack of enrichment of PU.1 targets in the leukaemic subtypes of the MILE study for the TRIB1 signature suggests that PU.1 control of TRIB1 expression may be specific to the normal cells of haematopoiesis.

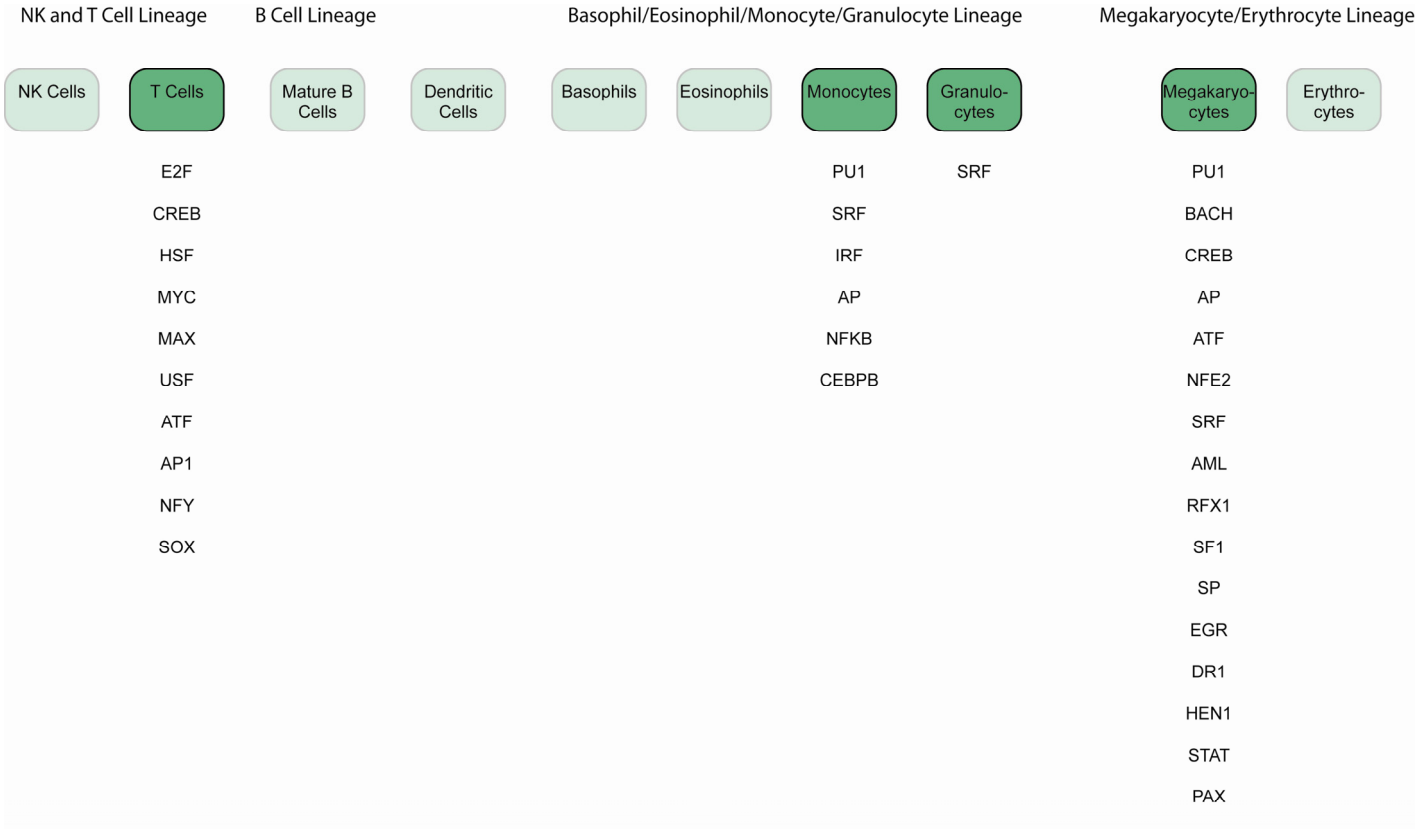

Figure 4.11: TFTs enriched in each of the different cell types and lineages of the haematopoietic system. GSEA analyses were performed for the TRIB1 signature for each of the cell types and cell lineages of the haematopoietic system (Novershtern et al., 2011). As for the MILE study the TFTs were then grouped based on type, TFTs types with more than one hit can be seen listed in the above below the relevant cell type ranked in order of negative enrichment score (NES). * denotes pathways that had only one hit in the GSEA analyses and are only listed if multiple pathway types were not found. Detailed tables with names of individual pathways enriched and p-values, q-value and NES can be found in the supplementary Table 4.X on the supplementary CD.

4.7 Analysis of the Relationship between TRIB1 and C/EBPα

GSEA analyses of the TRIB1 signature in the leukaemic subtypes revealed that this gene signature is enriched for genesets that are associated with the C/EBP transcription factors, chiefly C/EBPα (figures 4.9). In the AML patients with normal karyotype and other abnormalities the TRIB1 signature was found to be enriched for chemical and molecular perturbation genesets induced by C/EBP expression (figure 4.13 (a) and table 4.) and for genesets of TFTs of the C/EBP family of transcription factors including C/EBPα and C/EBPβ (figure 4.13(b)). These analyses suggest that TRIB1 expression in this leukaemia may be driven by C/EBPα expression. C/EBPα is a myeloid transcription factor mutated in approximately 9% of AML patients. This number can rise up to 20% for patients with a subtype of AML known as acute myeloblastic leukaemia with granulocytic maturation, this is AML with t(8;21) and is also known as the M2 subtype of leukaemia. Other leukaemias that arise from the granulocyte-lineage are also known to posses C/EBPα mutations. Mutations in C/EBPα result in aberrant function, not loss of function of the C/EBPα protein (Nerlov, 2004). As the bioinformatic data indicates that TRIB1 may be a target of the C/EBPα transcription factor we decided to try and validate this result at the bench.

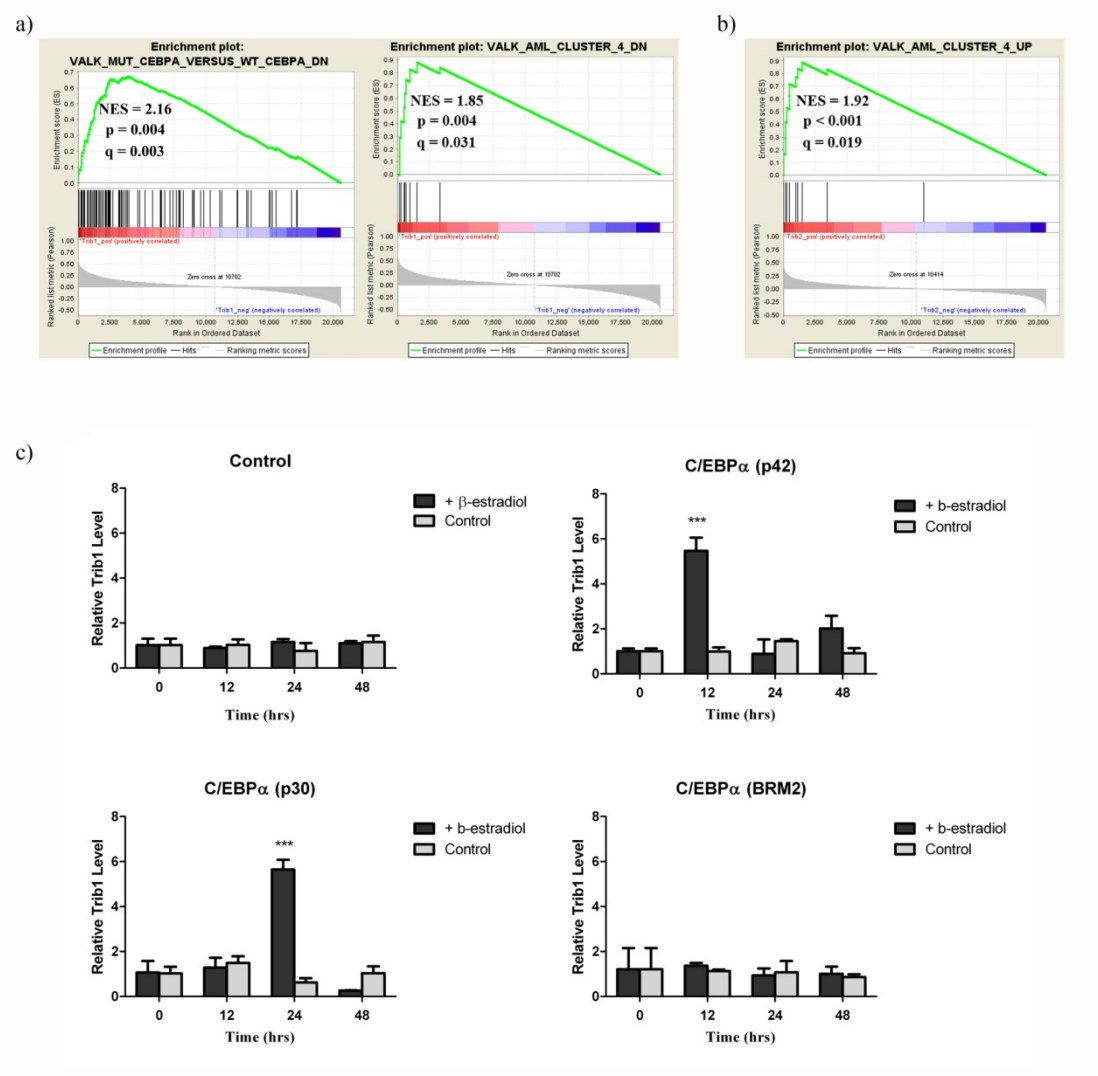

Figure 4.12: CEBPA induced TRIB1 expression in leukaemic cells and the TRIB1 signature is associated with normal C/EBPα expression. **a)** GSEA plots showing enrichment of the TRIB1 signature in AML leukaemic patient samples from the MILE study with genes up-regulated in AML patients with wildtype C/EBPα compared to patients with mutated C/EBPα status and down-regulated in Cluster 4 of the Valk AML patients samples (Valk et al., 2004). **b)** GSEA plots showing enrichment of the TRIB2 signature in AML leukaemic patient samples from the MILE study with genes up-regulated in Cluster 4 of the Valk AML patient samples (Valk et al., 2004). Normalised enrichment scores (NES), p-value and q-value (FDR) are indicated on each GSEA plot; a p-value below 0.05 and a q-value below 0.25 indicates that the result is significant. **c)** Induction of TRIB1 expression by the C/EBPα p42 and p30 isoforms in K562 cells. Stable K562 cells lines expressing inducible vectors expressing the p42, p30, BRM2 isoforms of C/EBPα or empty vector as a control were stimulated with β-estradiol or with control (ethanol). mRNA expression of TRIB1 measured at 12, 24 and 48 hours post-induction. Values are representative of three independent experiments. N = 3; Error bars indicate +/- SD.

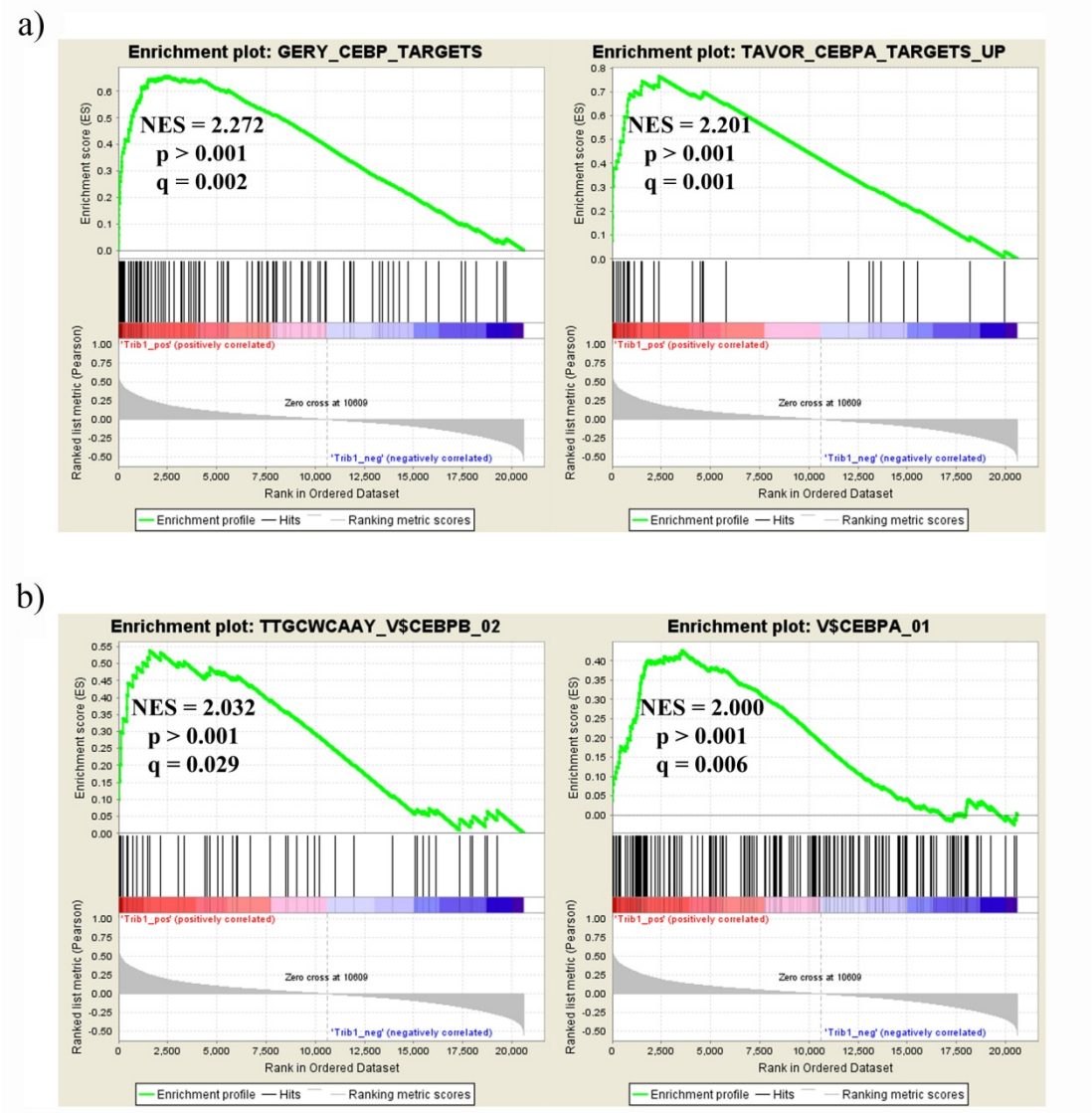

Figure 4.13: a) GSEA plots showing enrichment of the TRIB1 signature for genes induced by C/EBP expression in AML patient samples with normal karyotype and other abnormalities from the MILE study. **b)** GSEA plots showing enrichment of the TRIB1 signature for transcription factor targets of C/EBP transcription factors in AML patient samples with normal karyotype and other abnormalities from the MILE study. Normalised enrichment scores (NES), p-value and q-value (FDR) are indicated on each GSEA plot; a p-value below 0.05 and a q-value below 0.25 indicates that the result is significant.

Analyses of the TRIB1 signature in the AML patient samples of the MILE study revealed that the top 100 genes up-regulated in patients with wild-type C/EBPα compared to AML patients with mutated C/EBPα status are enriched in the signature (figure 4.12 (a)). Genes down-regulated in Cluster 4 of the Valk dataset are also enriched in the TRIB1 AML signature (figure 4.12 (a)). Cluster 4 of the Valk dataset is associated with a dysregulated C/EBPα signature and is linked with increased TRIB2 expression (Valk et al., 2004; Keeshan et al., 2006; Wouters et al., 2007). The TRIB2 signature is enriched for genes up-regulated in cluster 4 of the Valk dataset (figure 4.12 (b)). Analysis shown here indicates that TRIB1 expression is more highly enriched for genes associated with wildtype C/EBPα expression in AML compared to genes whose expression is associated with mutated C/EBPα in AML. The TRIB1 signature is also enriched with genes down regulation in patients with a dysregulated C/EBPalpha signature, which has been shown to be associated with elevated TRIB2 expression (Keeshan et al., 2006; Wouters et al., 2007) (figure 4.12 (b)). This data suggests that TRIB1 expression is closely associated with wild-type C/EBPα expression in leukaemia.

As the TRIB1 signature is associated with the wildtype C/EBPα signature in AML and is also enriched for C/EBP target genes many of the subtypes of leukaemia including normal karyotype AML (figure 4.13 (b)) we decided to ascertain if TRIB1 expression can be induced by C/EBPα expression in the cell. Since the TRIB1 signature is associated with wildtype and not mutant C/EBPα expression we also decided to analysis TRIB1 expression in response to the induction of mutated C/EBPα genes that mimic those found in patients. Two mutated forms of C/EBPα whose expression has been associated with the development of AML are the p30 and BRM2 forms (Porse et al., 2005; Hasemann et al., 2008; Kirstetter et al., 2008). The

p30 isoform of C/EBPα lacks the transactivation domain of the full length C/EBPα protein (C/EBPα p42). Frame-shift mutations giving rise to this isoform have been identified in patients (Nerlov, 2004). The BRM2 mutant of C/EBPα contains a mutation in the C-terminal basic region of C/EBPα (Porse et al., 2005). Both the p30 and BRM2 forms of C/EBPα can bind DNA (Lin et al., 1993; Porse et al., 2001), fail to induce granulocytic differentiation (D'Alo' et al., 2003; Keeshan et al., 2003; Wang et al., 2003) and have been observed in leukaemia (Nerlov, 2004).

Using a number of inducible K562 cell lines (a kind gift from Dr. Daniel G. Tenen (Boston, MA, USA)) regulation of TRIB1 expression in response to induction of the p42, the p30 and the BRM2 isoforms of C/EBPα was analysed. The multipotential K562-ER cells lines were derived from K562 cells, a CML cell line, by the stable transfection of a plasmid encoding an β-estradiol inducible C/EBPα-p42, C/EBPα-p30 or C/EBPα-BRM2 oestrogen receptor fusion protein (D'Alo' et al., 2003). Induction of both the full length C/EBPα protein (p42) and the truncated form of C/EBPα (p30) led to a transient increase in TRIB1 expression in these cells compared to control (figure 4.8 (c)). However expression of the BRM2 isoform of C/EBPα, a mutant form of C/EBPα which can bind DNA but is unable to repress E2F1 dependent transcription in the cell (Porse et al., 2001), did not increase TRIB1 expression (figure 4.8 (c)). The p30 isoform can induce TRIB1 expression though this induction occurs at a later time point than for the p42 isoform (figure 4.8 (c)). Although the p30 and p42 have been found to posses distinct regulatory roles, these two protein isoforms have also been reposted to similarly regulate a large number of genes (Wang et al., 2007). It has also been recently reported that the full length C/EBPα p42 protein can induce TRIB1 expression in K562 cells (Liss et al., 2013).

The BRM2 mutant of C/EBPα exhibits enhanced affinity for the E2F-Dinerization Partner (DP) complex and reduced affinity for DNA binding compared to wildtype C/EBPα. This increased affinity led to the dominant repression of transactivation by BRM2 by the E2F-DP complex. Knock-down of DP1 or E2F1, 3 or 4 led to the restoration of the binding of BRM2 to adipogenic target genes and induced their activation (Zaragoza et al., 2010). Reduced affinity for the TRIB1 promoter may explain the lack of induction of this gene by the BRM2 mutant.

The data presented suggests that TRIB1 is a target of wildtype myeloid C/EBPα transcription factor but not the C/EBPα BRM2 mutant in the cell. Though the p30 form of C/EBPα could still induce TRIB1 expression it did so in a time delayed manner which suggests potential perturbation of TRIB1 expression by the p30 isoform in the cell. This data is an interesting lead that validates the bioinformatic approach taken to try and identify potential regulators of TRIB1 expression in the cell.

Lukaemia

AML with Normal Karyotype and Other Abnormalities

NAME	SIZE	ES	NES	NOM p-val	FDR q-val
GERY_CEBP_TARGETS	109	0.658	2.273	> 0.001	0.002
TAVOR_CEBPA_TARGETS_UP	42	0.763	2.201	> 0.001	0.001
HALMOS_CEBPA_TARGETS_UP	40	0.701	2.120	> 0.001	0.003
HALMOS_CEBPA_TARGETS_DN	39	0.560	1.741	0.014	0.039

AML with Complex and Aberrant Karyotype

NAME	SIZE	ES	NES	NOM p-val	FDR q-val
HALMOS_CEBPA_TARGETS_UP	40	0.691	2.075	> 0.001	0.044
TAVOR_CEBPA_TARGETS_UP	42	0.702	2.029	> 0.001	0.027
GERY_CEBP_TARGETS	109	0.543	1.946	0.004	0.035
HALMOS_CEBPA_TARGETS_DN	39	0.532	1.653	0.026	0.134

CLL

NAME	SIZE	ES	NES	NOM p-val	FDR q-val
GERY_CEBP_TARGETS	109	0.617	2.207	> 0.001	0.006
HALMOS_CEBPA_TARGETS_UP	40	0.614	2.059	0.002	0.006
TAVOR_CEBPA_TARGETS_UP	42	0.672	1.985	0.002	0.011
HALMOS_CEBPA_TARGETS_DN	39	0.569	1.856	> 0.001	0.026

CML

NAME	SIZE	ES	NES	NOM p-val	FDR q-val
GERY_CEBP_TARGETS	109	0.574	2.042	> 0.001	0.041
TAVOR_CEBPA_TARGETS_UP	42	0.670	2.029	0.011	0.041
HALMOS_CEBPA_TARGETS_UP	40	0.626	1.937	0.024	0.061

MDS

NAME	SIZE	ES	NES	NOM p-val	FDR q-val
TAVOR_CEBPA_TARGETS_UP	42	0.671	1.975	0.004	0.017
GERY_CEBP_TARGETS	109	0.531	1.942	> 0.001	0.021
HALMOS_CEBPA_TARGETS_DN	39	0.609	1.877	0.004	0.034
HALMOS_CEBPA_TARGETS_UP	40	0.578	1.850	0.012	0.041

NBM

NAME	SIZE	ES	NES	NOM p-val	FDR q-val
GERY_CEBP_TARGETS	109	0.560	2.062	> 0.001	0.032
HALMOS_CEBPA_TARGETS_UP	40	0.582	1.767	0.008	0.177

Table 4.I: Table of C/EBP related genesets from the Chemical and Molecular Perturbations Geneset file (version 3.1) from the MSigDB enriched for the TRIB1 signature in the leukaemic subtypes of the MILE study.

Haematopoietic Lineages

Monocyte Lineage

NAME	SIZE	ES	NES	NOM p-val	FDR q-val
TAVOR_CEBPA_TARGETS_UP	42	0.727	2.033	> 0.001	0.197
HALMOS_CEBPA_TARGETS_UP	40	0.629	1.963	> 0.001	0.043
GERY_CEBP_TARGETS	105	0.491	1.883	> 0.001	0.037
HALMOS_CEBPA_TARGETS_DN	39	0.428	1.547	0.034	0.132

Granulocyte Lineage

NAME	SIZE	ES	NES	NOM p-val	FDR q-val
TAVOR_CEBPA_TARGETS_UP	42	0.721	2.136	> 0.001	0.050
HALMOS_CEBPA_TARGETS_UP	40	0.604	1.991	> 0.001	0.154
GERY_CEBP_TARGETS	105	0.485	1.870	> 0.001	0.119

Basophil Lineage

NAME	SIZE	ES	NES	NOM p-val	FDR q-val
TAVOR_CEBPA_TARGETS_UP	42	0.705	2.117	> 0.001	0.109
HALMOS_CEBPA_TARGETS_UP	40	0.487	1.782	0.002	0.128
HALMOS_CEBPA_TARGETS_DN	39	0.494	1.743	0.012	0.144
GERY_CEBP_TARGETS	105	0.435	1.740	0.012	0.145

Eosinophil Lineage

NAME	SIZE	ES	NES	NOM p-val	FDR q-val
TAVOR_CEBPA_TARGETS_UP	42	0.650	1.975	0.004	0.075
GERY_CEBP_TARGETS	105	0.449	1.787	0.004	0.097
HALMOS_CEBPA_TARGETS_UP	40	0.505	1.698	0.020	0.129
HALMOS_CEBPA_TARGETS_DN	39	0.479	1.686	0.014	0.136

Megakaryocyte Lineage

NAME	SIZE	ES	NES	NOM p-val	FDR q-val
TAVOR_CEBPA_TARGETS_UP	42	0.643	2.074	> 0.001	0.023
GERY_CEBP_TARGETS	105	0.507	1.872	> 0.001	0.032
HALMOS_CEBPA_TARGETS_DN	39	0.536	1.687	0.024	0.085
HALMOS_CEBPA_TARGETS_UP	40	0.483	1.598	0.035	0.123

T Cell Lineage

NAME	SIZE	ES	NES	NOM p-val	FDR q-val
TAVOR_CEBPA_TARGETS_DN	22	0.542	1.627	0.031	0.152

NKA Lineage

NAME	SIZE	ES	NES	NOM p-val	FDR q-val
TAVOR_CEBPA_TARGETS_UP	42	0.530	1.738	0.040	0.193
HALMOS_CEBPA_TARGETS_UP	40	0.474	1.630	0.028	0.250

Table 4.II: Table of C/EBP related genesets from the Chemical and Molecular Perturbations Geneset file (version 3.1) from the MSigDB enriched for the TRIB1 signature in the Haematopoietic lineages (Novershtern et al., 2011).

Though a number of haematopoietic cell lineages are enriched for C/EBP related genesets (table 4.II) only the monocyte lineage is enriched for TFTs of a C/EBP family member, in this case C/EBPβ, when analysed using the TRIB1 signature (figure 4.11). Both C/EBPα and C/EBPβ are myeloid transcription factors necessary for myeloid differentiation (Rosenbauer and Tenen, 2007; Huber et al., 2012). TRIB1 expression is induced by C/EBPα (figure 4.12 (c)) and, in the monocyte lineage, both TRIB1, C/EBPα and C/EBPβ show a similar expression pattern increasing as the cells differentiate into the mature monocyte cells (figure 4.14 (b)). In the granulocyte lineage TRIB1 and C/EBPα and C/EBPβ do not show as similar an expression pattern (figure 4.14 (a)). These data indicates that TRIB1 may be a target of the C/EBP family of transcription factors particularly during myeloid differentiation, specifically monocyte differentiation, in normal haematopoiesis.

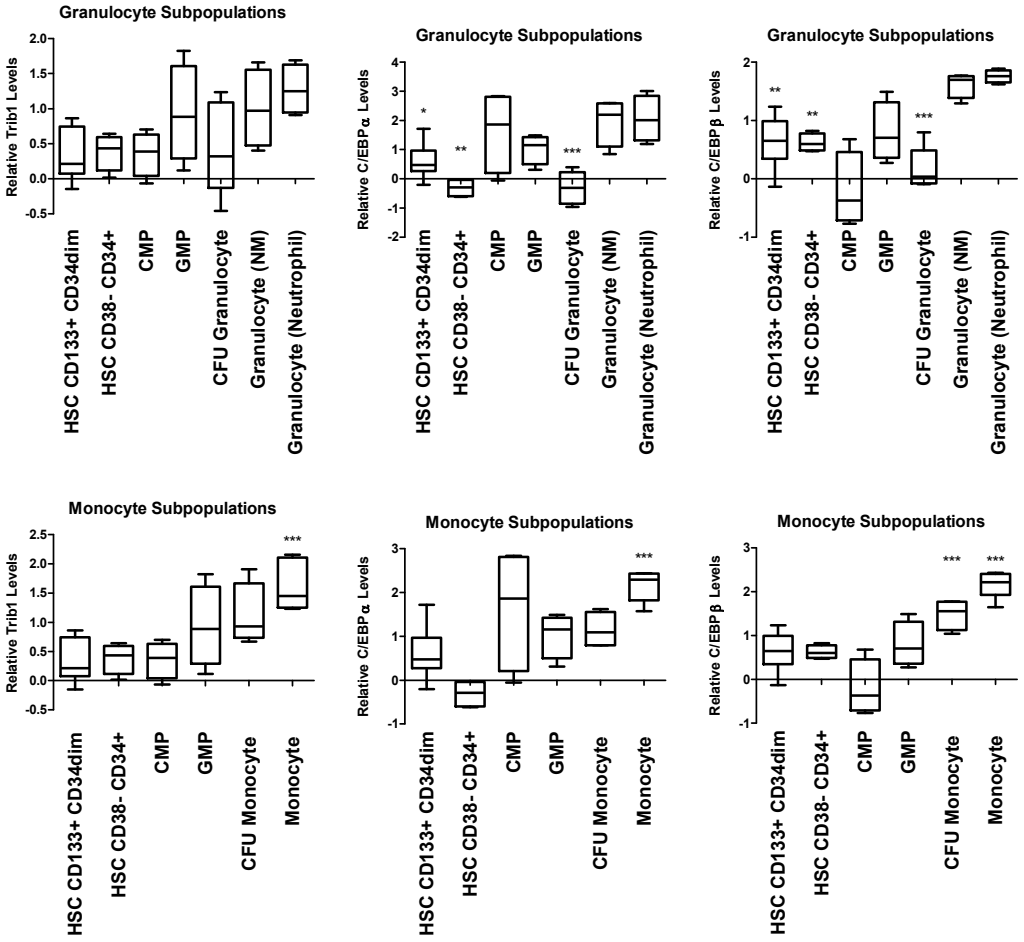

Figure 4.14: Expression profiles of TRIB1, C/EBPα and C/EBPβ expression in the granulocyte and monocyte lineages in normal haematopoiesis **a)** Expression levels in the granulocyte lineage. **b)** Expression levels in the monocyte lineage. Statistical analyses was carried out by performing a one-way ANOVA analyses followed by a Bonferroni's Multiple Comparison Test of each of the cell types versus the others using GraphPad Prism 5. A p-value below 0.05 indicated a significant difference in gene expression between cell types, p-value below 0.05 is represented by *, below 0.01 by ** and below 0.001 by ***.

4.8 Identification of Transcription Factor Targets (TFTs) associated with the TRIB2 signature in the leukaemic subtypes of the MILE study

T-ALL and ALL with t(1;19) have significantly higher levels of TRIB2 expression compared to the control group (figure 3.2), while ALL with t(12;21) and AML with (8;21) have significantly lower levels of TRIB2 expression (figure 3.2). As the T-ALL and ALL with t(1;19) subtypes show high TRIB2 expression our analysis is focused on these ALL subtypes. TFTs enriched in these two subtypes include targets of ETS, PU.1, E2F and STAT (figure 4.15). As increased TRIB2 expression has been linked to survival in CLL targets enriched in this subtype are also discussed (figure 4.16).

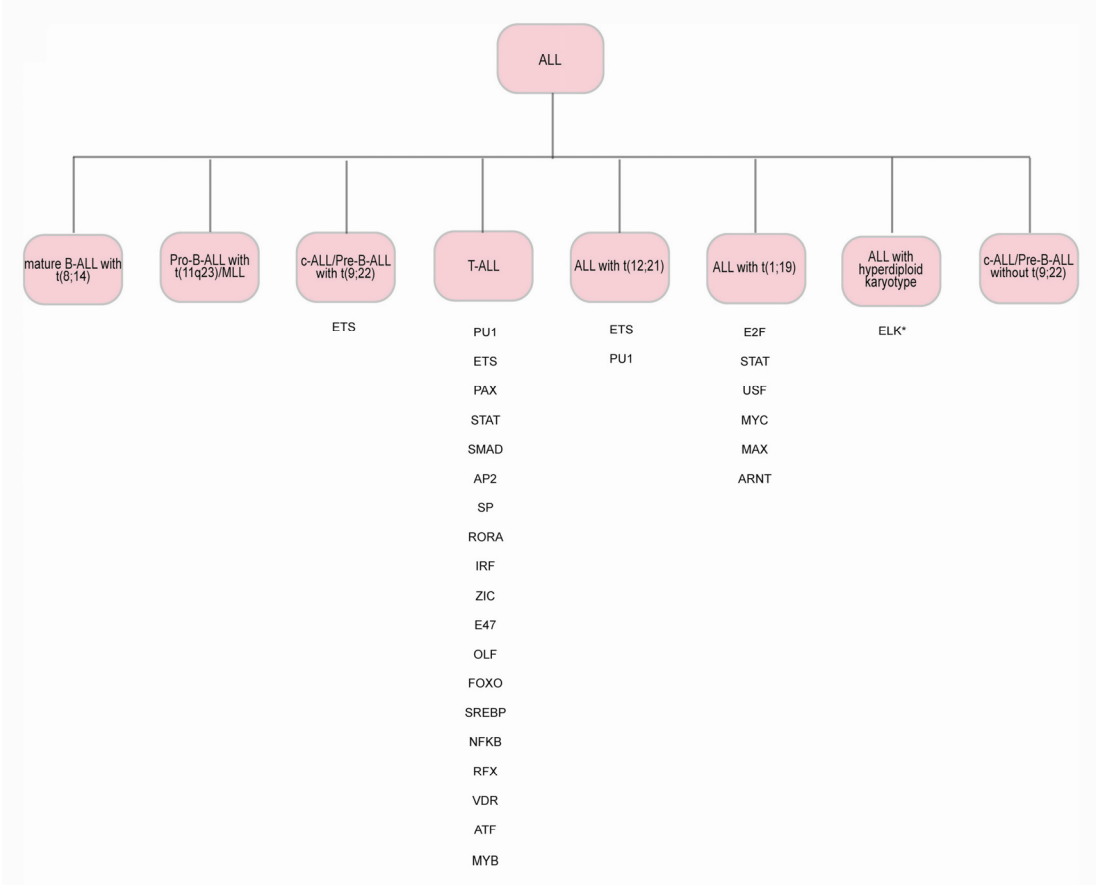

Figure 4.15: TFTs enriched in the ALL subtypes of the MILE Study for the TRIB2 signature. GSEA analyses were carried out for the TRIB2 signature in the leukaemic subtypes of the MILE. The TFTs were then grouped based on type. TFT types with more than one hit can be seen listed in the above below the relevant leukaemia subtype ranked in order of negative enrichment score (NES). Detailed tables with names of individual pathways enriched and p-values, q-value and NES can be found can be found in the supplementary Table 4.XI on the supplementary CD.

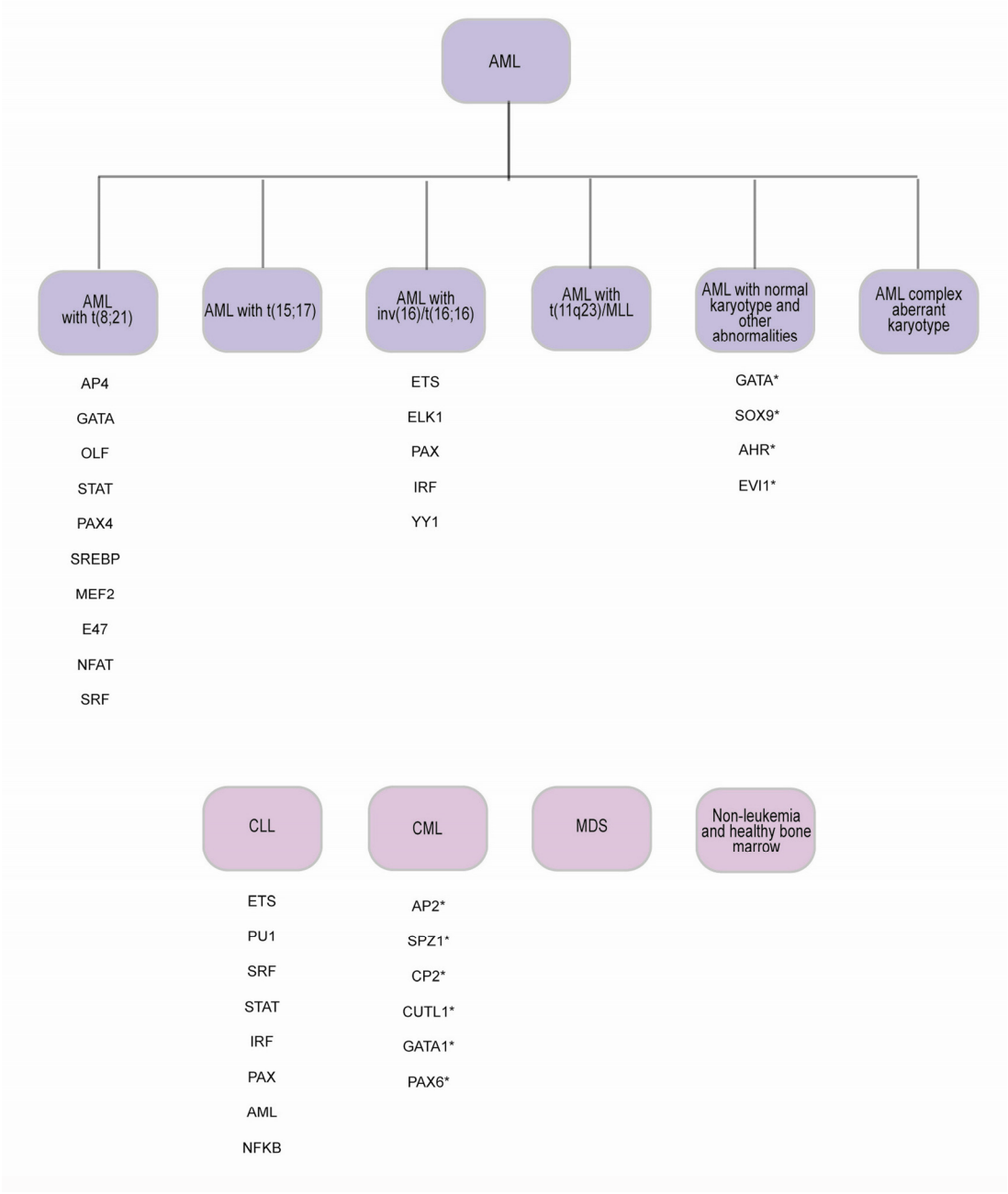

Figure 4.16: TFTs enriched in the AML subtypes, in CLL, CML, MDS and non-leukaemic patient samples of the MILE Study for the TRIB2 signature. GSEA analyses were carried out for the TRIB2 signature in the leukaemic subtypes of the MILE. The TFTs were then grouped based on type. TFT types with more than one hit can be seen listed in the above below the relevant leukaemia subtype ranked in order of negative enrichment score (NES). Detailed tables with names of individual pathways enriched and p-values, q-value and NES can be found can be found in the supplementary Table 4.XI on the supplementary CD.

Targets of the ETS (ETS1 and ETS2) transcription factors are enriched for the TRIB2 signature in T-ALL and ALL with t(12;21) and CLL (figures 4.15 and 4.16). ETS is a member of the ETS transcription factor family is made up of proteins that share a conserved winged helix-turn-helix DNA binding domain (ETS domain) that recognises unique DNA sequences containing GGAA/T (Ets binding sites, EBS). ETS proteins may also contain the Pointed (PNT) domain, necessary for protein–protein interaction (Seth et al., 1992; Oikawa and Yamada, 2003). The ETS family plays an important role in cancer as well as in many biological processes including control of cellular haematopoiesis, proliferation, differentiation, apoptosis, tissue remodelling and angiogenesis (Sementchenko and Watson, 2000; Oikawa and Yamada, 2003; Dittmer, 2003; Hsu et al., 2004; Seth and Watson, 2005).

TFTs of PU.1 are also enriched in the T-ALL, as well as ALL with t(12;21) and CLL leukaemic subtypes of the MILE study for the TRIB2 signature (figures 4.15 and 4.16). PU.1 has been shown to be a tumour suppressor in myeloid leukaemia, however an increase in the levels of PU.1 expression in early T cells leads to the development of T cell leukaemia (Kastner and Chan, 2008). TFTs for PU.1 are enriched in T-ALL for the TRIB2 signature where TRIB2 expression is significantly higher than the control group (figure 3.2) suggests that PU.1 expression may be driving an increase in TRIB2 levels which may play a role in the development of T-ALL.

Both T-ALL, ALL with t(1;19) and CLL are all enriched with TFTs of the STAT family of transcription factors for the TRIB2 signature (figures 4.15 and 4.16). The STAT family of transcription factors are activated by the phosphorylation of JAK proteins upon the binding of cytokines or interferons to their receptors, phosphorylation allows the STAT proteins to dimerise and translocate into the

nucleus. Here the STAT proteins can modulate the expression of target genes (Leonard and O'Shea, 1998). The JAK-STAT pathway plays an important role in immunity, immunodeficiency and in cancer (O'Shea et al., 2013). Dysregulation of the JAK kinases is associated with leukaemia and lymphoma (Chen et al., 2012) and 40% of large granular lymphocytic leukaemia's have mutated STAT3 (Koskela et al., 2012).

Targets of the E2F family of transcription factors are enriched in ALL with t(1;19) for the TRIB2 signature (figure 4.15). This subtype of leukaemia possesses the highest TRIB2 expression (figure 3.2). The E2F family of transcription factors have a well established and critical role in cell cycle progression (Dyson, 1998). It has also been shown that E2F is involved in the integration of cell cycle progression with DNA repair, replication, and the G_2/M checkpoints (Ren et al., 2002). E2F1 has been identified as a strong regulator of apoptosis after DNA damage in all types of human cancer (Engelmann and Pützer, 2010). However increased E2F1 expression is also associated with cancer and metastasis (Tsantoulis and Gorgoulis, 2005; Engelmann and Pützer, 2012). Increased E2F1 activity has been connected with both AML (Pulikkan et al., 2010) and with paediatric T-cell lymphoblastic leukaemia and lymphoma (Bonn et al., 2012), two forms of leukaemia which TRIB2 is also associated with (Keeshan et al., 2006; Wouters et al., 2007) and data published by us linking increased TRIB2 expression to paediatric T-ALL with activating NOTCH1 expression (see appendix A)). As this is an interesting observation the link between E2F and TRIB2 has been further explored in chapter 5.

TFTs of the PAX family of transcription factors were found to be enriched with the TRIB2 signature in the T-ALL and CLL subsets of leukaemia (figures 4.15 and 4.16). The PAX genes are critical in the development of different tissues during

embryogenesis and have long been associated with cancer (Chi and Epstein, 2002; Robson et al., 2006; Wang et al., 2008). PAX5, TFTs of which are enriched in the T-ALL, and CLL patient samples (supplementary Table 4.XI on the supplementary CD), is crucial for instigating and preserving B cell lineage specificity (Hagman and Lukin, 2006; Nutt and Kee, 2007) by repressing the expression of genes involved in commitment to other lineages all the while activating B cell specific genes (Nutt et al., 1999; Cobaleda et al., 2007; Schebesta et al., 2007). PAX5 haploinsufficiency has been confirmed to synergize with STAT5 to initiate ALL (Heltemes-Harris et al., 2011). TFTs of both PAX5 and STAT5 are enriched in the T-ALL patient samples for the TRIB2 signature (figure 4.15) indicating that control of TRIB2 expression by these two transcription factors may be a factor in the development of T-ALL.

GSEA using the chemical and molecular perturbation geneset file available for the Molecular Signatures Database (v3.0 MSigDB) revealed a number of genesets enriched in the various leukaemic subtypes of the MILE study that complement the results of the GSEA using the TFT genesets (v3.0 MSigDB) for the TRIB2 signature. Overall, there were few genesets overall enriched for the TRIB2 signature across the MILE study and in the cells and lineages of haematopoiesis (Supplementary Tables 4.VIII and 4.X on the supplementary CD).In the CLL patient samples, which were found enrich for TFTs of the PAX family (figure 4.16) and have a subset of patients with high TRIB2 expression (figure 3.3) there is also enrichment for the geneset LUI_THYROID_CANCER_PAX8_PPARG_UP (supplementary Table 4.VI on the supplementary CD). A geneset that contains the top up-regulated genes distinguishing between follicular thyroid carcinoma samples by the presence or absence of the PAX8-PPARG fusion protein (Lui et al., 2005).

As TRIB2 expression is associated with a subset of AML with normal karyotype (figure 3.3) (Keeshan et al., 2006) enrichment of the TFT for the TRIB2 signature was analysed in these samples. Single genesets of TFTs of GATA, SOX9 and EVI1 were enriched in this leukaemic subtype (figure 4.16). Though only one set for each of these TFT were enriched these data still point to a potential regulatory role played by these transcription factor in enhanced TRIB2 expression in this AML subtype. Enrichment of a geneset of GATA targets, for example, is of interest as GATA transcription factors are master regulators of haematopoiesis and have recently been linked to hematologic malignancies (Bresnick et al., 2012). Particularly GATA-2, inherited mutations of which have been associated with a predisposition to developing AML (Ostergaard et al., 2011).

4.9 Identification of Transcription Factor Targets (TFTs) associated with the TRIB2 signature in the cellular compartments and the cellular lineages of the human haematopoietic system

GSEA analyses of the cellular compartments and the cellular lineages of the human haematopoietic system showed that only the NK cells (figure 4.17) and the monocyte and granulocytic lineages (figure 4.18) of the haematopoietic system are enriched for more than one geneset of TFTs for the same transcription factors.

Targets of the E2F family of transcription factors are enriched in ALL with t(1;19) for the TRIB2 signature (figure 4.15), the subtype of leukaemia that possesses the highest TRIB2 expression (figure 3.2). NK cells (figure 4.17) which also have high TRIB2 expression relative to the other cellular compartments of the haematopoietic system (figure 3.2), and the monocytic lineage (figure 4.18), are enriched for more

than one geneset of TFTs of the E2F transcription factor family for the TRIB2 signature. Therefore, E2F transcription factors may be associated with high TRIB2 expression in ALL with t(1;19) and the NK cells. The geneset KALMA_E2F1_TARGETS, which consists of DNA replication genes up-regulated in a Rat-1a (a fibroblast) cell line by expression of E2F1(Kalma et al., 2001) is enriched in the NK cell compartment of the haematopoietic cells (supplementary table 4.VIII on the supplemental CD). The geneset EGUCHI_CELL_CYCLE_RB1_TARGETS, which contains RB1 target genes involved in cell cycle regulation that can be down-regulated by doxorubicin treatment only in cells expressing RB1 (Eguchi et al., 2007), is also enriched for the TRIB2 signature in the NK cells This geneset consists of genes that cannot be down-regulated when RB1 is silenced and include the oncogene Epithelial Cell Transforming 2 (ECT2) which is a target of E2F1 (Kalma et al., 2001). The RB1 protein is involved of the repression of E2F protein activity preventing E2F transcription factors from activating their target genes through phosphorylation of the E2F proteins (Dyson, 1998).

Early myeloid cell maturation is characterised by increasing levels of TRIB2 expression as the cells develop from HSC to CMP to GMP cells. TRIB2 expression levels are then reduced as the cells mature into monocytic cells to their lowest levels of any of the haematopoietic cellular compartments (figure 3.11). The monocyte lineage is enriched for TFTs of the E2F family (figure 4.18). Control of the TRIB2 cellular levels may be critical to monocytic cell development which may be achieved though transcription factors such as the E2F family.

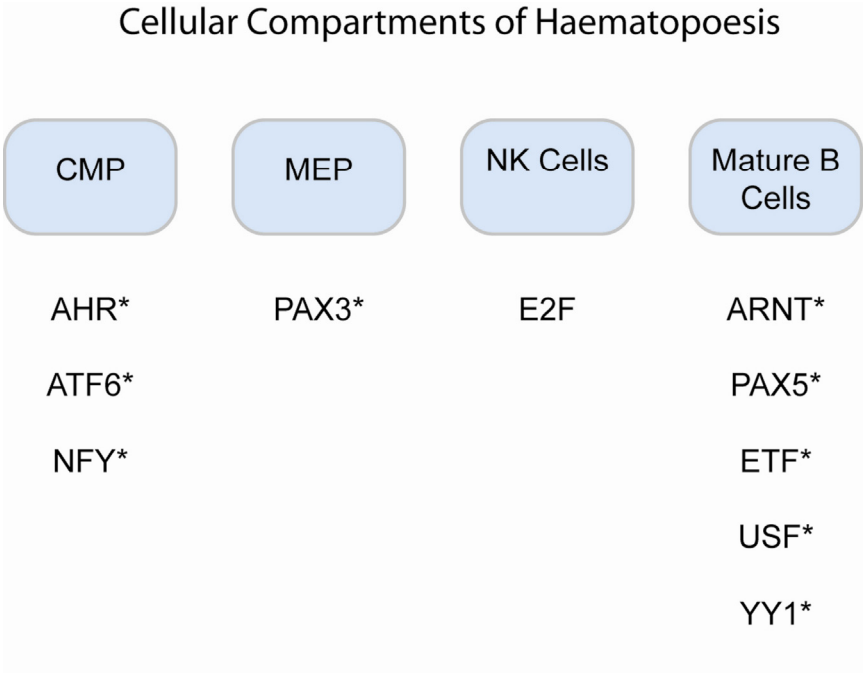

Figure 4.17: TFTs enriched in each of the different cell types of the haematopoietic system. GSEA analysis was performed for the TRIB2 signature for each of the cell types of the haematopoietic system (Novershtern et al., 2011). As for the MILE study the TFTs were then grouped based on type, TFT types with more than one hit can be seen listed in the above below the relevant cell type ranked in order of negative enrichment score (NES). * denotes pathways that had only one hit in the GSEA analyses and are only listed if multiple pathway types were not found. Detailed tables with names of individual pathways enriched and p-values, q-value and NES can be found in the supplementary Table 4.XII on the supplementary CD.

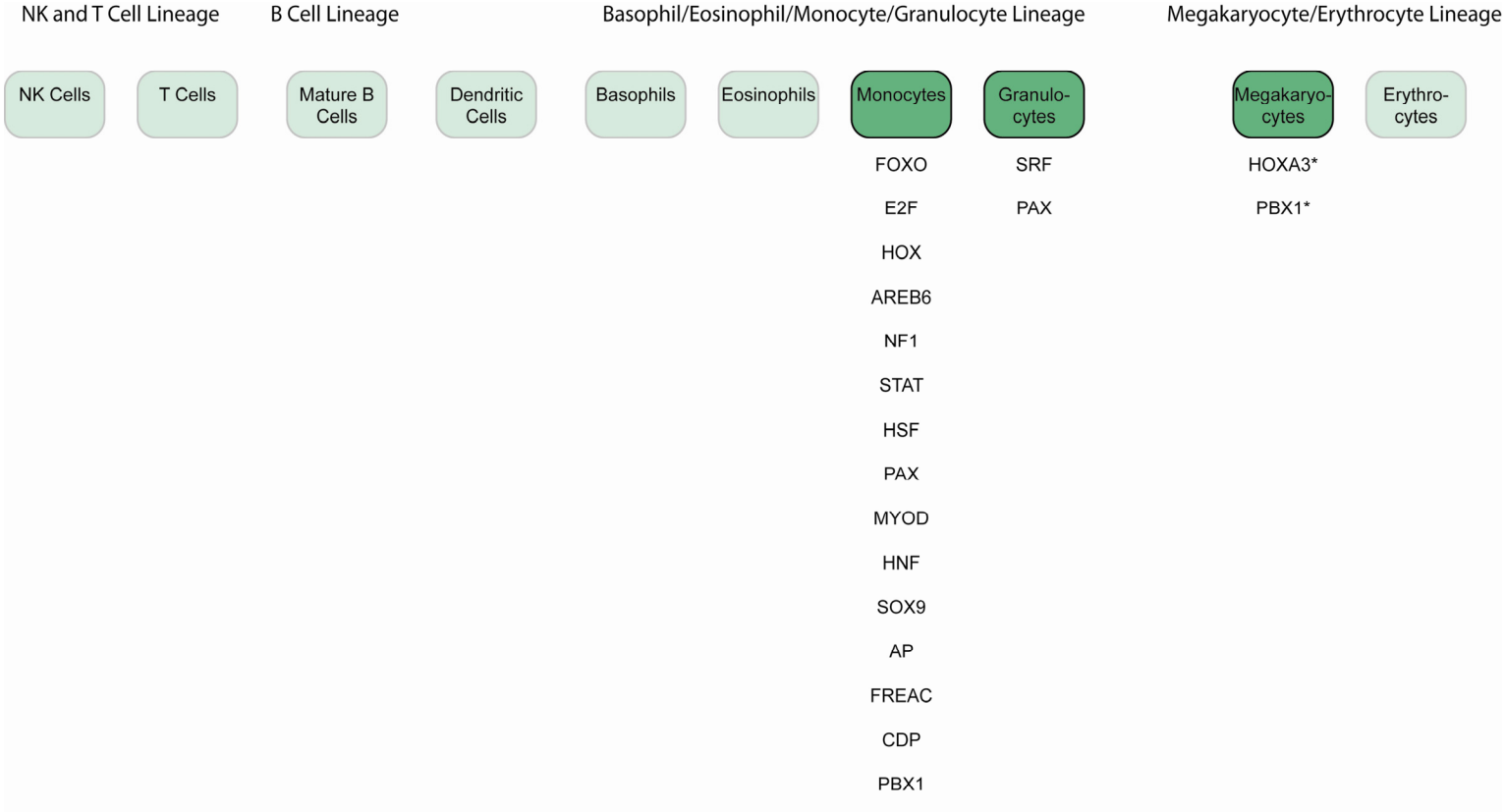

Figure 4.18: TFTs enriched in each of the different lineages of the haematopoietic system. GSEA analysis was performed for the TRIB2 signature for each of the cell lineages of the haematopoietic system (Novershtern et al., 2011). The TFTs were then grouped based on type, TFT types with more than one hit can be seen listed in the above below the relevant cell type ranked in order of negative enrichment score (NES). * denotes pathways that had only one hit in the GSEA analyses and are only listed if multiple pathway types were not found. Detailed tables with names of individual pathways enriched and p-values, q-value and NES can be found in the supplementary Table 4.XII on the supplementary CD..

The monocytic cell lineage is enriched for TFTs of the PAX, STAT and PBX transcription factor families. The PAX and STAT transcription factor families are both enriched in a number of leukaemic subtypes of the MILE study for the TRIB2 signature (figures 4.15 and 4.16). Here we see evidence that they may play a role in TRIB2 expression for normal cellular development (figures 4.17 and 4.18). The PBX transcription factor, TFTs of which are only found enriched for the TRIB2 signature in the monocyte and megakaryoctye lineages of haematopoiesis (figure 4.18). They are important transcription factors that interact with the Hox family of proteins and form heterodimers (Shen et al., 1997; Wu et al., 2006) and with Meis1 (Shen et al., 1999).

Enrichment for HOX TFTs is also found for the TRIB2 signature in the monocyte and megakaryoctye lineages of haematopoiesis (figure 4.18). While TFTs of the HOX family, which are implicated in the development of leukaemia (Eklund, 2011; Magli et al., 1997), are not found in the leukaemic subtypes of the MILE study for the TRIB2 signature (figures 4.15 and 4.16), enrichment is found for the TRIB1 signature AML with normal karyotype and other abnormalities (figure 4.9). Both TRIB1 and TRIB2 have been shown to cooperate with HOX family members in the development of myeloid leukaemia (Jin et al., 2007; Keeshan et al., 2008b). Here we have evidence for regulation of TRIB2 expression by the HOX family of transcription factors in normal haematopoiesis, specifically in the monocytic and megakaryocytic cell lineages. The granulocytic lineage is also enriched with the chemical and molecular perturbations geneset FERRANDO_HOX11_NEIGHBORS (Supplementary Table 4.VIII found on the supplementary CD) which consist of genes that are nearest neighbours of HOX11, based on the close agreement of their expression profiles with that of HOX11 in paediatric T-ALL (Ferrando et al., 2002).

Discussion

GSEA enrichment analyses for the TRIB1 and TRIB2 signatures in both leukaemia and the normal cells of haematopoiesis revealed a large number of pathways, TFTs and leukaemic signatures enriched for both these signatures. Many similar pathways are enriched for both the TRIB1 and TRIB2 signature in both the leukaemic subtypes of the MILE study and in the cellular compartments and lineages of haematopoiesis. Overlapping pathways include the T cell and T cell Co-Stimulation Pathways, TLR pathways, apoptosis Pathways, B cell pathways and immune system signalling pathways as well as the Her/EerB and Wnt signalling pathways discussed above. While the TRIB1 is overwhelmingly enriched for the TLR signalling pathways, the MAPK signalling pathways and immune system signalling pathways, the T cell and T cell Co-Stimulation Pathways, apoptosis pathways and B cell pathways are more highly enriched for the TRIB2 signature across the MILE study and in the cells and lineages of haematopoiesis. These pathways reflect the conclusion that TRIB1 may be considered a myeloid associated gene while TRIB2 is more strongly associated with the lymphoid compartment. Enrichment for similar pathways indicated that TRIB1 and TRIB2 may play similar roles in the cell, however the vast differences in the level and frequency of enrichment of these pathways shows that TRIB1 and TRIB2 are uniquely expressed and may play both overlapping and unique roles in the cell.

G-protein coupled receptor pathways (Lattin et al., 2007), NF-κB pathways (Vallabhapurapu and Karin, 2009) and TLR pathways (Tapping, 2009) have all been associated with immune system function. As these and many of the pathways enriched for the TRIB1 signature suggesting that TRIB1 expression is functionally

involved in immune system function. TRIB1 expression has previously been associated with TLR signalling (Yamamoto et al., 2007) and TLR signalling pathways are enriched for the TRIB1 signature in the AML samples with normal karyotype and other abnormalities and in the monocytic cell lineage

Unique enrichment of pathways, such as the Notch signalling pathway in the monocytic lineage, for the TRIB1 signature in the different myeloid lineage suggests that TRIB1 is associated with cell specific pathways development during haematopoiesis, and specifically during myelopiesis. Analysis of the TRIB1 signature suggests that it may convey an APL like signature in non-APL AML leukaemia patient samples, as it is enriched for genes highly expressed in APL. Pathways such as the TLR and NF-κB pathways are enriched for the TRIB1 signature across both the MILE leukaemic subtypes and in the cell compartments and lineages of haematopoiesis are associated with cancer (Basith et al., 2012; DiDonato et al., 2012). Induction of aberrant TRIB2 expression in liver cancer by the Wnt/β-Catenin has recently been reported (Wang et al., 2013). This is a pathway that has been previously associated with leukaemia and cancer (Anastas and Moon, 2013; Memarian et al., 2012; Okuhashi et al., 2011). The Wnt/β-Catenin signalling pathway was enriched for both the TRIB1 (AML with normal karyotype and other abnormalities) and TRIB2 (T-ALL and CLL) signatures. Supporting this, targets of β-catenin 1 gene and/or of the Wnt gene are enriched across both the leukaemic subtypes and in the cellular compartments and the lineages of the cells of haematopoiesis for these two signatures. This data associates TRIB1 and TRIB2 signalling in leukaemia with this pathway.

TRIB2 is strongly associated with T cell signalling by enrichment of the T cell and T cell Co-Stimulation Pathways. This links TRIB2 expression in leukaemia,

particularly in normal karyotype AML with a T cell signature. This has previously been reported (Wouters et al., 2007). Association of TRIB2 with the apoptotic signal is also of interest as it suggests that TRIB2 may play a role in the determination of cell survival.

TRIB2 is a target of Notch1 in T-ALL cells (Keeshan et al., 2006; Wouters et al., 2007) and is linked to Tri2 to Notch signalling in T-ALL (published in the British Journal of Haematology, 2012) (See appendix A) , a leukaemia where activating mutations of Notch1 are frequent (Weng et al., 2004). Very little is known about the regulation of both TRIB1 and TRIB2 expression. TRIB2 expression is induced by miR-98 (S. Xie et al. 2012), Notch1 (Hannon et al., 2012; Keeshan et al., 2006) and is inhibited by miR-511 and miR-1297 (Zhang et al., 2012). However, to date, TRIB1 has not been identified as a direct target of any transcription factor and the regulation of both TRIB1 and TRIB2 expression in both normal and leukaemic cells is not clear. Analyses of both the leukaemic subsets of the MILE study and the cellular compartments and lineages of haematopoiesis identified a number of potential transcription factors that may regulate TRIB1 and TRIB2 expression in both normal and leukaemic cells. Many of these transcription factors have been shown to be involved both in haematopoiesis and in leukaemogenesis adding weight to the idea that TRIB1 and TRIB2 expression is important in haematopoiesis and dysregulation of this expression may lead to leukaemia.

One of the transcription factor families which were identified as a possible regulator of TRIB1 expression is the C/EBP family. While both TRIB1 and TRIB2 can degrade C/EBPα and induce myeloid leukaemia in the cell (Keeshan et al., 2006; Jin et al., 2007; Dedhia et al., 2010) only TRIB2 is associated with the dysregulated C/EBPα signature. These studies the bioinformatic finding, here, that TRIB1 is a

potential transcription factor target of C/EBPα (and possible other members of the C/EBP family, particularly C/EBPβ).

Differences in the genesets enriched for the TRIB1 or TRIB2 signatures point to individual and unique transcription factor targets regulating TRIB1 and TRIB2 expression. However since some overlap in enrichment of TFTs occurs for the two signatures, just as for the canonical pathways, it is clear that some overlap in the regulation of TRIB1 and TRIB2 expression may occur depending on cell type and, perhaps, context.

In conclusion, the Tribbles genes are tissue and cell specific and are associated with both overlapping and cell specific pathways and gene signatures as assessed by the analysis presented here. The large range of diverse pathways and TFTs enriched for both the TRIB1 and TRIB2 signatures discovered here suggest that there is much to discover regarding the different functions and regulation of Tribbles genes in normal and malignant haematopoiesis

Chapter 5

E2F Regulation of TRIB2 Expression

This work was published in Blood 23(15):2389-400; April 10th 2014 under the title "Regulation of TRIB2 by an E2F1-C/EBPalpha feedback loop in AML cell proliferation" Loveena Rishi, Maura Hannon, Mara Salomè et al. (Appendix B).

Introduction

Over-expression of Trib2 has been shown to cause AML in retroviral mouse models (Keeshan et al., 2006) and TRIB2 expression has also been linked to other cancers including lung (Grandinetti et al., 2011; Zhang et al., 2012), liver (Wang et al., 2013) and melanoma (Zanella et al., 2010). To date very little is known about how TRIB2 expression is induced both in the normal cells of haematopoiesis and in leukemic cell. Trib2 expression has been shown to be up-regulated by miR-98 in early lesions of the large arteries of type 2 diabetic rats (Xie et al., 2012) and is co-regulated by FoxA1 and TCF4 in liver cancer cells (Wang et al., 2013). TRIB2 expression is also down-regulated by miR-511 and miR-1297 in lung cancer (Zhang et al., 2012). In leukaemia Trib2 was found to be a Notch1 target in a murine T-ALL cell line (Keeshan et al., 2006).

The E2F family of transcription factors consists of eight members, E2F1 – E2F8, with two protein isoforms, E2F3a and E2F3b, produced from the E2F3 gene. The E2F family is associated with transcription, proliferation and apoptosis and is conventionally split into two groups. E2F1 – E2F3 are known as activating E2F as they activate the transcription of E2F target genes and E2F4 – E2F8 are known as repressing E2F as they repress the expression of E2F target genes (DeGregori and Johnson, 2006). E2F1 plays a critical role in inducing apoptosis in response to DNA damage particularly in human cancers (Engelmann and Pützer, 2010). Although E2F1 is commonly viewed as a tumour suppressor, E2F1 gene amplification and/or abnormal E2F1 gene expression is found in many types of human cancer. Increased E2F1 gene expression is often associated with high-grade tumours or metastases and unfavourable prognosis (Engelmann and Pützer, 2012).

The activator E2F transcription factors also play a role in haematopoiesis. E2F1 deficient mice show a slight increase in the number of T cells in their thymus due to impaired

apoptosis (Field et al., 1996). E2F2 deficient mice show defective development of erythrocytes and an increase in the number of T lymphocytes (Murga et al., 2001). E2F1 and E2F2 deficient mice show a block in B cell differentiation beyond the pre B cell stage as well as impaired maturation of the erythrocytes (Li et al., 2003). E2F1, E2F2 and E2F3 deficient mice have decreased numbers of myeloid cells (Trikha et al., 2011; Matsumoto and Nakayama, 2013). Repression of E2F dependent transcription by C/EBPα is necessary for granulocytic differentiation (Rosenbauer and Tenen, 2007) and dysregulated E2F1 activity has been associated with both AML (Pulikkan et al., 2010) and with paediatric T-cell lymphoblastic leukaemia and lymphoma (Bonn et al., 2012).

Earlier analysis (chapter 4) showed that targets of the E2F family of transcription factors were enriched in ALL with t(1;19) for the TRIB2 signature (figure 4.10 (a)), the subtype of leukaemia that possesses the highest TRIB2 expression (figure 3.1(a)). NK cells, which also have high TRIB2 expression relative to the other cellular compartments of the haematopoietic system (figure 3.1(b)), are also enriched for more than one geneset of TFTs of the E2F transcription factor family (figure 4.11 (a and b)) for the TRIB2 signature, as is the monocytic lineage (figure 4.11 (a and c)).

In this study we show that TRIB2 is a target of the E2F family of proteins. The TRIB2 promoter region was found to contain a number of potential E2F transcription factor binding sites. Luciferase assays indicated that E2F1, E2F3 and E2F4 activate the Trib2 promoter and, in the case of E2F1, this activation was dependent on the ability of E2F1 to bind DNA. Using deletion mutants of the Trib2 promoter and site directed mutagenesis the region on the Trib2 promoter to which E2F1 binds was narrowed down and chromatin immunoprecipitation assays revealed that E2F1, E2F2 and E2F3 proteins bind the TRIB2 promoter. Over-expression of E2F1 in E2F1 deficient cells resulted in an increase in TRIB2 expression. This

data suggests that TRIB2 is a direct target of E2F1 which may play a role in the regulated expression of TRIB2 in leukaemogenesis and haematopoiesis.

Results

5.1 Analyses of TRIB2 expression in leukemic cell lines

TRIB2 mRNA expression varies in both the normal cells of haematopoiesis and across the many leukemic subtypes as assessed in microarray datasets (figure 3.1(a and b)). Screening of TRIB2 mRNA expression in both murine (figure 5.1 (a)) and human leukemic cell lines (figure 5.1 (b)) by reverse transcription-polymerase chain reaction (RT-PCR) analysis showed wide variation in TRIB2 expression. In the murine cells Trib2 mRNA expression was highest in primary spleen cells with the next highest expression levels found in the primary bone marrow cell samples. This is consistent with the finding that TRIB2 expression is highest in the T cell compartment of haematopoiesis (figure 3.1(b)) as the spleen is rich in T-cells. Of the murine cell lines tested, the fibroblast cell line 3T3 expresses the highest level of Trib2. TRIB2 mRNA expression also varies across the human leukemic cell lines. Expression was found to be high in a number of cell lines including the K562, HL-60 and NB4 cells (figure 5.1 (b)). Together these data indicate that TRIB2 expression levels vary depending on cell type both in murine cells and in human leukemic cell lines.

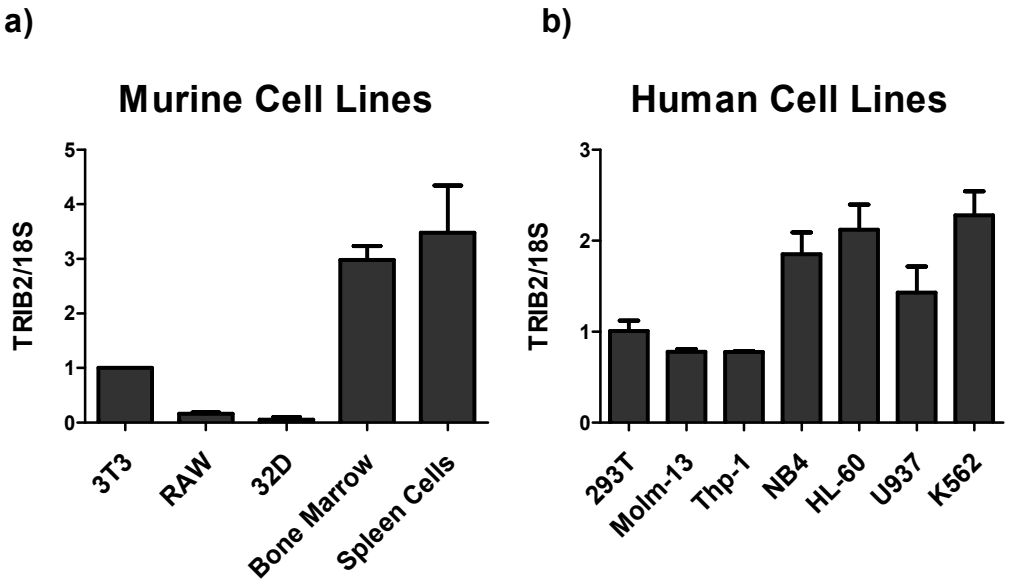

Figure 5.1: TRIB2 expression in human and murine cell lines. **a)** Trib2 expression is highest in primary murine spleen and bone marrow cells and lowest in the 32D murine cell line. Trib2 mRNA expression levels were analysed by real-time PCR, error bars are +/- standard deviation from duplicate samples and values are representative of experiments performed in duplicate. **b)** TRIB2 expression levels are highest in the K562 human cell line and lowest in the Thp-1 human cell line. TRIB2 mRNA expression levels were analysed by real-time PCR and normalised to 18S expression, error bars are +/- standard deviation from duplicate samples and values are representative of experiments performed in duplicate.

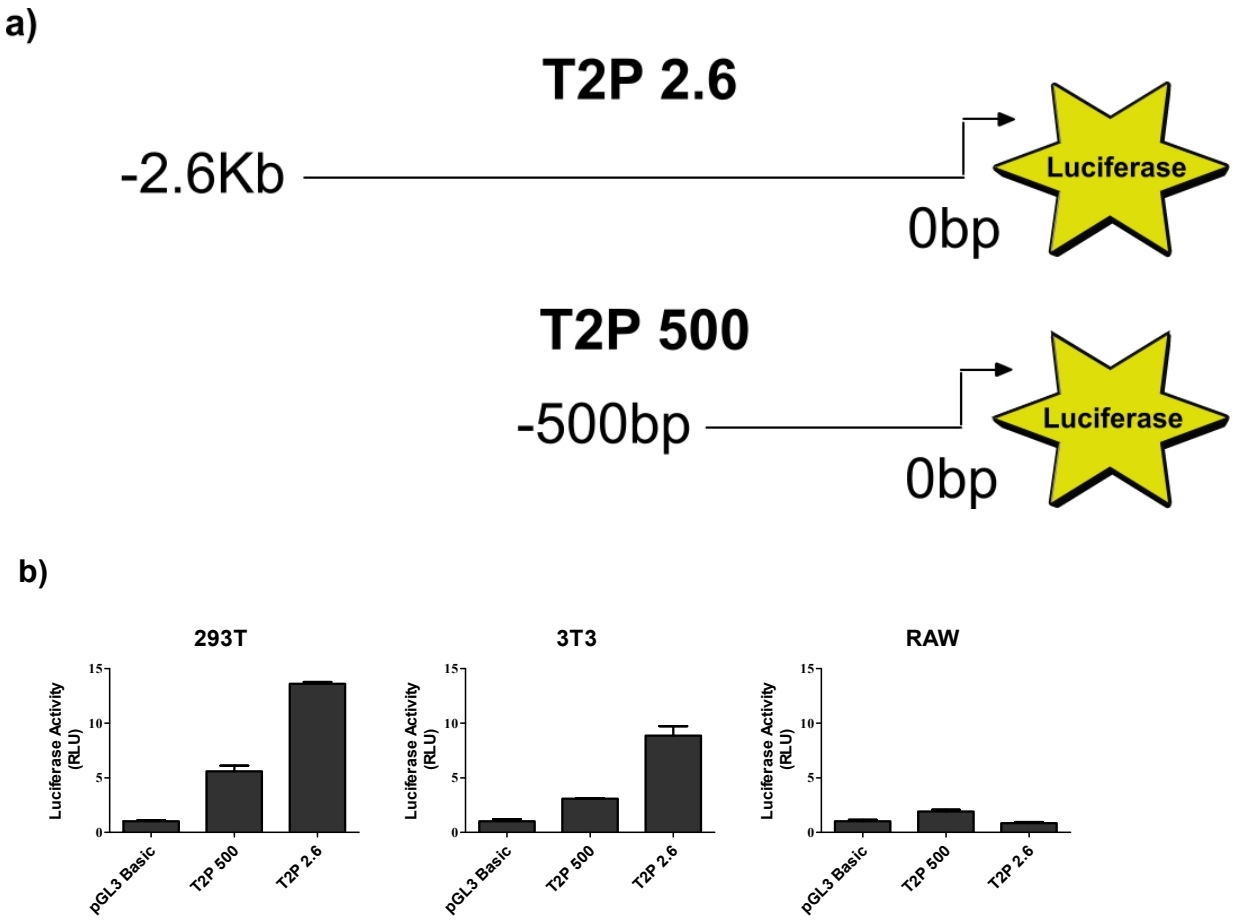

Figure 5.2: Trib2 luciferase reporter activity in different cell lines. **a)** Schematic of the T2P 500 and T2P 2.6 luciferase promoter constructs each of which contain a portion of the Trib2 promoter region. **b)** The T2P 500 and T2P 2.6 luciferase promoter constructs are active in both the 293T and 3T3 cell lines but not the RAW cell line. Luciferase reporter activity for both constructs was normalised to pGL3 Basic in each of the cell lines. Values are representative of experiments performed, at least, in duplicate. Error bars indicate +/- SD.

Two regions of the murine Trib2 promoter, -500bp and -2.6kb upstream of the transcription start site of Trib2, were cloned into a luciferase reporter vector (pGL3 Basic) (figure 5.2 (a), cloned by Karen Keeshan). These constructs are called the T2P 500 and the T2P 2.6 respectively. These constructs were each transfected into 293T cells, a human embryonic kidney cell line, murine 3T3 cells, a fibroblast cell line and RAW cells, a murine macrophage cell line and their activities were compared to control (pGL3 Basic). Both reporter constructs are active in 293T and 3T3 cell (figure 5.2 (b)). The T2P 2.6 is more active than the T2P 500 in these two cell types (figure 5.2 (b)) indicating that the T2P 2.6 possess addition transcription factor target sites that are actively inducing luciferase expression. Neither of the luciferase promoter constructs is active in the RAW cell line, a murine monocytic cell line. Trib2 expression was found to be low in the RAW cell line (figure 5.1 (a)) and in the monocytic compartment of the human haematopoietic cells (figure 3.1(b)). Low Trib2 expression and the inactivity of the Trib2 reporter constructs indicate that Trib2 promoter is not activated in this cell line.

5.2 Analyses of the promoter region of Trib2 and identification of transcription factor regulating Trib2 expression using the luciferase assay system.

Using the Transcription Element Search System (TESS), a web tool for predicting transcription factor binding sites in DNA sequences (Schug and Overton, 1997) a large number of potential transcription factor binding sites were identified within the Trib2 promoter region (figure 5.3 and Table 5.I). Potential binding sites include binding sites for the E2F and C/EBP families of transcription factors. One of the E2F binding sites identified was an E2F-p107 transcription factor binding site. p107 is a member of the retinoblastoma (Rb) family and a repressor of E2F1, 2 and 3 activities in cycling cells. p107 binds to E2F

proteins, preferentially E2F4, at their transcription factor binding sites repressing their activity. Phosphorylation of p107 releases it from E2F allowing it to activate the promoter region of its target genes (Di Fiore et al., 2013; Komori, 2013). The other E2F binding sites only show evidence for E2F binding though this does not rule out the potential of binding partners of co-occupy at these sites.

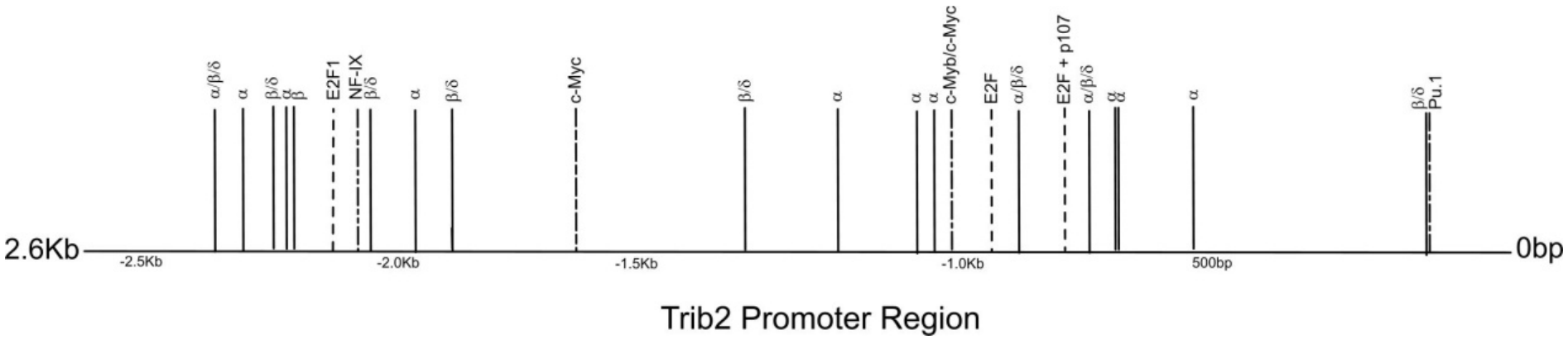

Figure 5.3: Schematic of potential transcription factor binding sites identified on the Trib2 promoter region using TESS. α indicates a potential C/EBPα binding site, β indicates a C/EBPβ binding site and δ indicates a potential C/EBPδ binding site. Full details of the potential binding sites can be found in table 5.I.

Factor	Model	Beg	Sns	Len	Sequence	L_a	$L_{a/}$	Lq	Ld
C/EBPalpha	R02132 ()	-718	R	10	CTTTGCAATT	20.00	2.00	1.00	0.00
E2F	R08804 ()	-943	R	8	CCCGGAAA	16.00	2.00	1.00	0.00
E2F-1	R09581 ()	-2145	N	8	CTTGGCCT	16.00	2.00	1.00	0.00
NF-1X	R02209 ()	-2100	R	14	TTGGCNNNNNKCCR	16.00	1.14	1.00	0.00
C/EBP	I00272 (C/EBP)	-766	R	9	TTTTGCAAT	13.26	1.47	1.00	0.00
C/EBPbeta	R02170 ()	-2214	R	7	TTYCCAG	13.00	1.86	1.00	0.00
C/EBPalpha	R02132 ()	-1050	N	8	GTGGWWWG	13.00	1.62	1.00	0.00
C/EBPalpha	R02132 ()	-576	R	8	CWWWCCAC	13.00	1.62	1.00	0.00
c-Myc	R02893 ()	-1018	N	6	CAGTTG	12.00	2.00	1.00	0.00
E2F + p107	R08844 ()	-811	N	6	TCGCGG	12.00	2.00	1.00	0.00
PU.1	R09480 ()	-1701	R	6	ACGGTG	12.00	2.00	1.00	0.00
c-Myc	R04413 ()	-149	N	6	TTCCTC	12.00	2.00	1.00	0.00
C/EBPalpha/C/EBP	R02132 ()	-895	R	9	MTTRCNNMA	11.00	1.22	1.00	0.00
C/EBPalpha/C/EBP	R04247 ()	-766	N	9	TKNNGYAAK	11.00	1.22	1.00	0.00
C/EBPalpha/C/EBP	R04247 ()	-2360	R	9	MTTRCNNMA	11.00	1.22	1.00	0.00
PU.1	I00047 (PU.1)	-149	N	6	TTCCTC	10.93	1.82	1.00	0.00
C/EBPalpha	R02132 ()	-1082	R	5	GCAAT	10.00	2.00	1.00	0.00
C/EBPalpha	R02132 ()	-714	R	8	GCAAT	10.00	2.00	1.00	0.00
C/EBPalpha	R01446 ()	-1225	N	5	ATTGG	10.00	2.00	1.00	0.00
C/EBPalpha	R02132 ()	-2310	N	5	ATTGC	10.00	2.00	1.00	0.00
C/EBPalpha	R01446 ()	-2230	R	5	CCAAT	10.00	2.00	1.00	0.00
C/EBPalpha	R01446 ()	-1993	R	5	CCAAT	10.00	2.00	1.00	0.00
C/EBPbeta/C/EBPdelta	R02216 ()	-895	R	9	MTTNCNNMA	10.00	1.11	1.00	0.00
C/EBPbeta/C/EBPdelta	R02216 ()	-766	N	9	TKNNGNAAK	10.00	1.11	1.00	0.00
C/EBPbeta/C/EBPdelta	R02216 ()	-150	R	9	MTTNCNNMA	10.00	1.11	1.00	0.00
C/EBPbeta/C/EBPdelta	R02216 ()	-2360	R	9	MTTNCNNMA	10.00	1.11	1.00	0.00
C/EBPbeta/C/EBPdelta	R02216 ()	-2251	R	9	MTTNCNNMA	10.00	1.11	1.00	0.00
C/EBPbeta/C/EBPdelta	R02216 ()	-2078	N	9	TKNNGNAAK	10.00	1.11	1.00	0.00
C/EBPbeta/C/EBPdelta	R02216 ()	-1928	N	9	TKNNGNAAK	10.00	1.11	1.00	0.00
C/EBPbeta/C/EBPdelta	R02216 ()	-1392	N	9	TKNNGNAAK	10.00	1.11	1.00	0.00

Table 5.I: TESS analyses results of the 2.6Kb promoter region of Trib2 (region of promoter cloned into T2P 2.6). Displayed on this table are the results for a number of transcription factors that were found to have potential binding sites in the Trib2 promoter region. Model is the site string or weight matrix used to pick the site, Beg gives the location of the start of the site in the sequence (0 is the transcriptional start site for Trib2), Sns is the sense of the site (N is normal, R is reverse compliment), Len is the length of the site, Sequence is the sequence of the binding site matched to the model, La is the Log-likelihood score (the higher the better), La/ is the La /Len (higher is better, maximum value is 2.00), Lq is the La/L_M (L_M is the maximum La possible for the site model, higher is better and the maximum values if 1.00) and Ld is the L_M – La (0 is the best value, higher values indicate a sores match). Table is sorted by La values followed by La/ values. In order to remove less robust binding sites only results with a La score above 10 were examined.

Having identified these putative transcription factor binding sites a reporter assay using the T2P 500 and T2P 2.6 was performed in order to determine if any of these sites can activate or repress the Trib2 promoter. The T2P 500 and T2P 2.6 reporters were transfected along with plasmids expressing the relevant transcription factors (figure 5.4). Two-way ANOVA analyses of the activation of the luciferase reporters (pGL3 Basic, T2P 500 and T2P 2.6) by the transcription factors showed that this activation is significant. A Bonferroni test, used to counteract false positives due to multiple comparisons, was performed after the two-way ANOVA to determine if the difference between the activation of each luciferase reporter with each transcription factor compared to control was significant. Significance testing of all subsequent luciferase data was performed in the same way. This revealed that there is a significant decrease in reporter activity between the T2P 2.6 transfected with control (pcDNA3) and the same reporter transfected with C/EBPα, C/EBPβ, NFIX, PU.1 or Myc. The T2P 2.6 contains potential binding sites for each of these transcription factors. Though the T2P 500 also contains the PU.1 potential binding site only the T2P 2.6 is affected by this transcription factor. The T2P 2.6 may contain addition unidentified PU.1 binding sites or cooperation between PU.1 and additional transcription factors may be affecting the activation of the T2P 2.6. The Myc transcription factor represses the activity of the T2P 2.6; however there is a significant increase in T2P 500 activity when compared to T2P transfected with pcDNA3. The T2P 500 does not contain any identified Myc binding sites. Myc may be interacting with additional transcription factors that drive Trib2 expression which are repressed by Myc binding in the larger T2P 2.6 reporter.

While C/EBPα, C/EBPβ, NFIX, PU.1 and Myc all repress the activation of the T2P 2.6, E2F1 activates this promoter construct (figure 5.4). Activation of the T2P 2.6

reporter was significant compared to control (T2P 2.6 transfected with pcDNA3) as determined by the bonferroni post two-way ANOVA analyses. The T2P 2.6 reporter contains three potential E2F binding sites (figure 5.3 and table 5.I).

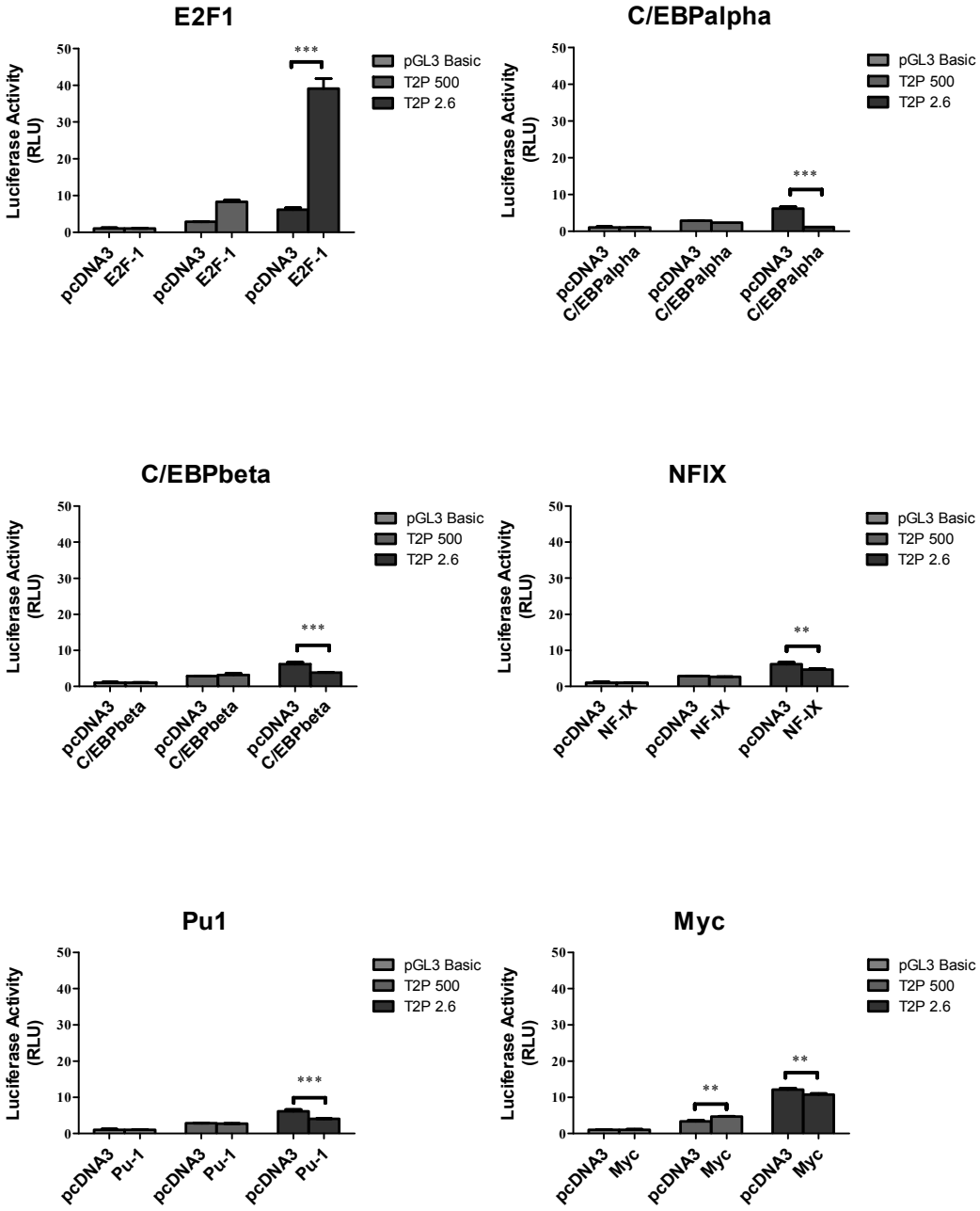

Figure 5.4: Transcription factors effect on Trib2 promoter activity. E2F1 can activate the T2P 2.6 promoter construct while other transcription factors such as C/EBPα and C/EBPβ can repress T2P 2.6 promoter activity in 3T3 cells. The T2P 500 and T2P 2.6 promoter constructs along with expression plasmids for a number of transcription factors were transfected into 3T3 cells. Values are normalised to relevant promoter transfected with control (pcDNA3) and are representative of experiments performed in duplicate. Error bars indicate +/- SD of duplicate samples. Significance was calculated by two-way ANOVA analyses with bonferroni post test. A p-value below 0.05 is considered significant, p-value below 0.05 is represented by *, below 0.01 by ** and below 0.001 by ***.

5.3 Identification of site of E2F1 binding within the Trib2 promoter region

In order to determine if the E2F1 transcription factor was directly binding the Trib2 promoter the promoter assay was repeated using a mutated form of E2F1, E2F1 E132. E2F1 E132 is DNA binding deficient that contains a point mutation that prevents the E2F1 protein from binding to the DNA but does not affect the interaction between E2F and its binding partners (Hsieh et al., 1997; Phillips et al., 1997). Unlike wild-type E2F1 the E2F1 E132 was unable to activate the Trib2 promoter region (figure 5.5) indicating that E2F1 must bind to the Trib2 promoter region in order to activate the Trib2 promoter.

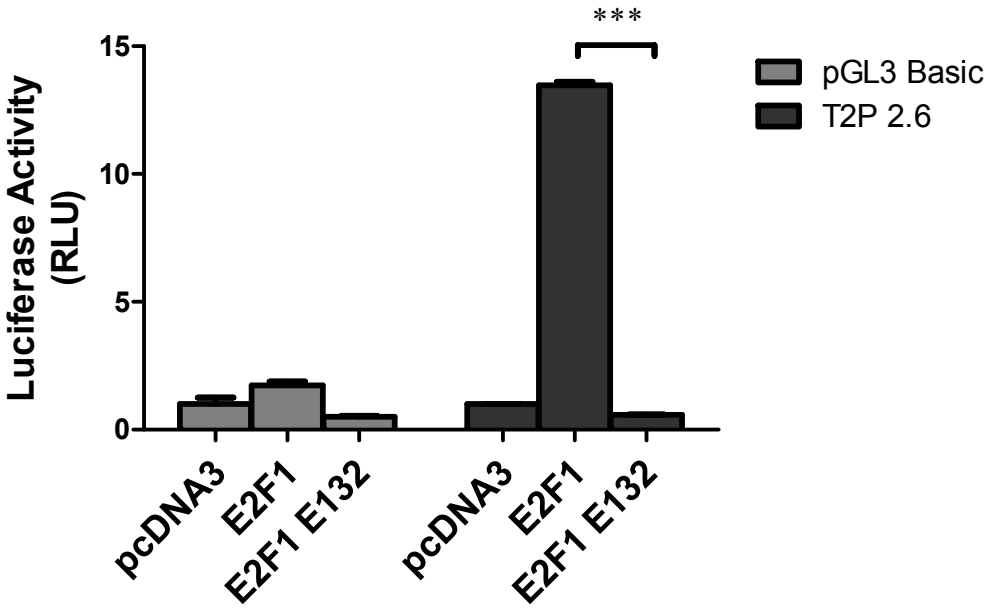

Figure 5.5: E2F1 activation of the Trib2 promoter is dependent on its ability to bind DNA. The DNA binding deficient E2F1 mutant E2F1 E132 is unable to activate the T2P 2.6 reporter. The pGL3 Basic and T2P 2.6 promoter constructs along with expression plasmid of the relevant transcription factors were transfected into 3T3 cells. Values are normalised to the relevant promoter transfected with control (pcDNA3) and are representative of experiments performed in duplicate. Error bars indicate +/- SD of duplicate samples. Significance was calculated by two-way ANOVA analyses with bonferroni post test. A p-value below 0.05 is considered significant, p-value below 0.05 is represented by *, below 0.01 by ** and below 0.001 by ***.

Analyses of the Trib2 promoter region indicated that there are three potential E2F1 binding sites within the T2P 2.6 Trib2 promoter construct (figure 5.3 and table 5.I). In order to determine the region of E2F1 binding within the Trib2 promoter region a number of deletion mutants of the Trib2 promoter and promoter constructs with mutations of the E2F binding sites were generated (figure 5.6 (a) and 5.7 (a)).

A number of deletion mutants of the Trib2 promoter construct were generated (figure 5.6 (a)). These deletion mutants were termed the T2P 927 (contains 927bp upstream of the Trib2 transcriptional start point), the T2P 963 (contains 963bp upstream of the Trib2 transcriptional start point, E2F1 binding site at -811bp on the Trib2 promoter region is mutated). These deletion mutants each contained one of the potential E2F1 binding sites on the Trib2 promoter (figure 5.6 (a)) and, unlike the T2P 500 which contains no potential E2F1 binding sites, both deletion mutants were significantly more active than control (figure 5.6 (b)). Activation of these two deletion mutants was significantly less than that of the full length T2P 2.6 reporter indicating that in the presence of either E2F binding site E2F1 could not fully restore promoter activity (figure 5.6 (b)). This suggests that both of these E2F binding sites are important for promoter activity.

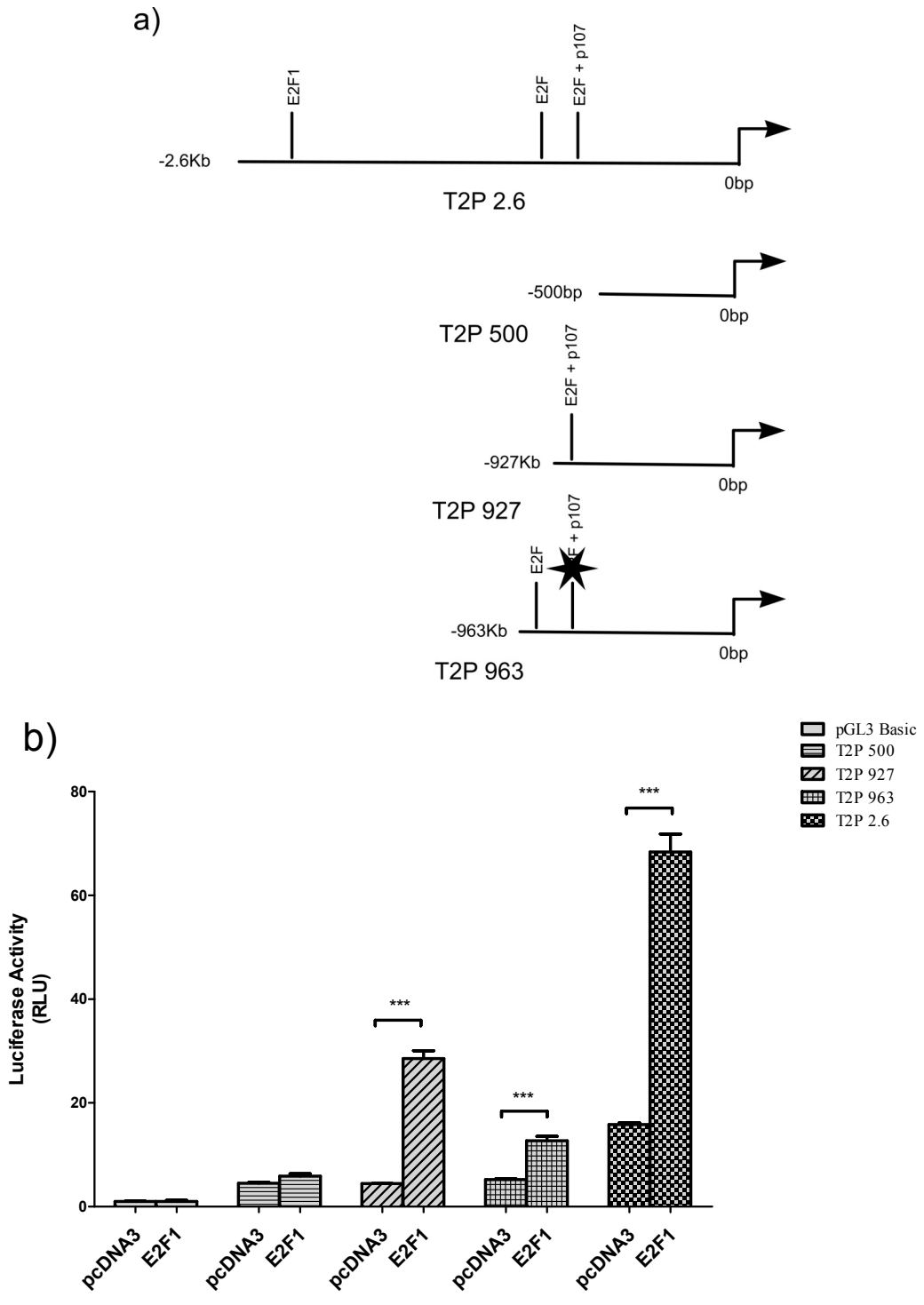

Figure 5.6: a) Schematic of T2P deletion mutants generated from the T2P 2.6 reporter. **b)** E2F1 activation of the T2P deletion mutants is significantly less than activation of the full length T2P 2.6 promoter construct. Values are normalised to the relevant promoter transfected with control (pcDNA3) and are representative of experiments performed in duplicate. Error bars indicate +/- SD of duplicate samples. Significance was calculated by two-way ANOVA analyses with bonferroni post test. A p-value below 0.05 is considered significant, p-value below 0.05 is represented by *, below 0.01 by ** and below 0.001 by ***.

In order to further narrow down the location of the E2F1 binding site in the Trib2 promoter a number of mutants of the T2P 2.6 reporter were generated (figure 5.7 (a)). These mutants were called T2P Mutant A (T2P Mut. A), T2P Mutant B (T2P Mut. B) and T2P Mutant C (T2P Mut. C). In each of the mutants one of the potential E2F binding sites identified in the Trib2 promoter region is mutated (figure 5.7 (a)). Another mutated T2P 2.6 reporter construct was generated called T2P Double Mutant (T2P DM). In this reporter both the potential E2F binding sites mutated in Mutant B and Mutant C are mutated (figure 5.7 (a)).

The luciferase reporter assay using these constructs revealed that while there was no reduction in promoter activation compared to the T2P 2.6 for the mutants T2P Mut. A and T2P Mut. C (figure 5.7 (b)). There is significant reduction in reporter activity for the T2P Mut. B compared to T2P 2.6 (figure 5.7 (b)). The T2P Double Mutant (DM) also showed significant reduction in activation versus the T2P 2.6 (figure 5.7 (b)). The T2P DM also shows slightly less activation than the T2P Mut. B (figure 5.7 (b)).

Together these data indicate that E2F1 is binding the Trib2 promoter within an E2F responsive region that contains both the potential E2F binding site located at -943bp (mutated in T2P Mut B) and –811bp (mutated in T2P Mut C.) upstream of the transcriptional start site of the Trib2 gene. No change in promoter activity for the T2P Mut. A (figure 5.7 (b)) indicates that E2F is not acting through this potential binding site (figure 5.7 (b)). Though mutation of the potential E2F binding site in the T2P Mut. C reporter does not affect reporter activity (figure 5.7 (b)) mutation of both potential E2F binding sites found in T2P Mut. B and Mut. C. results in lower activation of the T2P DM reporter compared to T2P Mut. B.

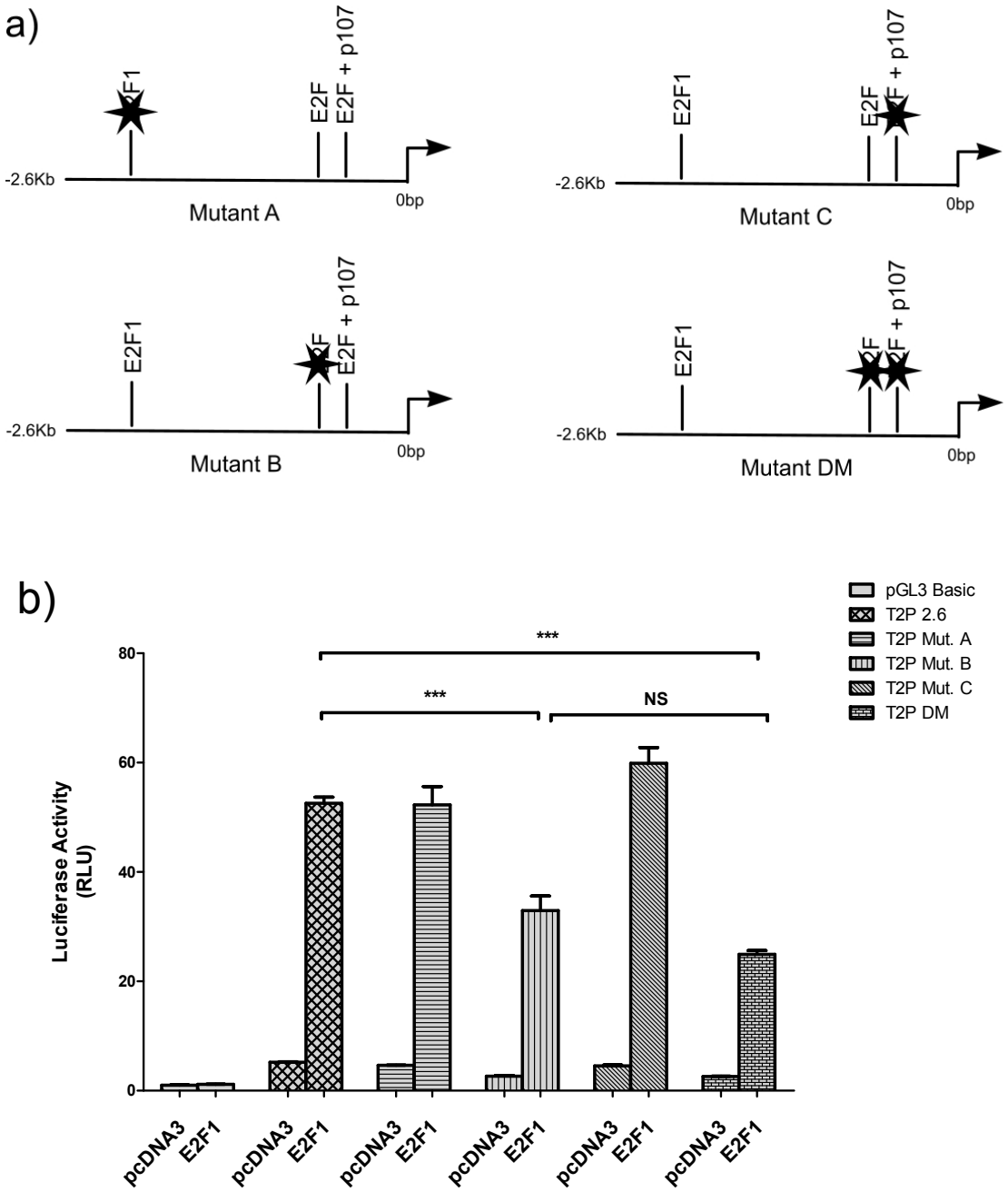

Figure 5.7: a) Schematic of mutants of the T2P 2.6 generated by site directed mutagenesis. **b)** E2F1 activation of the T2P Mut. B and T2P DM is significantly less than that of the T2P 2.6. Values are normalised to the relevant promoter transfected with control (pcDNA3) and are representative of experiments performed in duplicate. Error bars indicate +/- SD of duplicate samples. Significance was calculated by two-way ANOVA analyses with bonferroni post test. A p-value below 0.05 is considered significant, p-value below 0.05 is represented by *, below 0.01 by ** and below 0.001 by ***.

5.4 Activation of the Trib2 promoter by other members of the E2F family of transcription factors

E2F1 is a member of the E2F family of transcription factors which contains seven other members, E2F2 – E2F8. These transcription factors have each been shown to bind the E2F consensus sequence (DeGregori and Johnson, 2006).E2F1 – E2F3 are often referred to as the activating E2Fs, as they activate E2F target genes while E2F4 – E2F8 are referred to as repressor E2Fs, as they repress the expression of E2F target genes (DeGregori and Johnson, 2006) though it has been reported that E2F4 can also activate gene expression (Lee et al., 2011).

It has been reported that E2F1 – E2F5 can induce the same target genes to a slightly different degree and in a slightly different manner (Nevins et al., 1997). Since E2F1 can activate the Trib2 promoter (figure 5.4), a luciferase reporter assay was performed in order to assess the ability of other members of the E2F family to activate the Trib2 promoter. Using the T2P 2.6 reporter luciferase assays were performed with the E2F1, E2F3, E2F4 and E2F5 transcription factors. As well as E2F1, E2F3 and E2F4 were found to significantly activate the Trib2 reporter (figure 5.9) where as E2F5 could not.

Luciferase assays show that E2F1, E2F3 and E2F4 activate the Trib2 promoter (figure 5.9) and the E2F binding site was narrowed down to an E2F responsive region within the Trib2 promoter containing the potential E2F binding site located at -943bp and –811bp of the Trib2 promoter (figure 5.6 (b) and 5.7 (b)). Chip assays were performed in myeloid leukemic cells (K562), in order to determine if E2F1 – E2F5 of the E2F family of transcription factors can bind to the TRIB2 promoter. K562 cells were used for the Chip assay as they show the highest level of TRIB2

expression compared to many leukemic cell lines (figure 5.2 (a)). However K562 cells are a human cell line and the luciferase assay was performed using a promoter construct containing the murine Trib2 promoter. Analyses of the human TRIB2 promoter region revealed that this promoter contains two potential E2F binding sites (table 5.II)**.** Alignment of the TRIB2 promoter region revealed the E2F binding site mutated in T2P Mut. C is conserved in both the human and murine TRIB2 promoter region (figure 5.8 (highlighted in pink)). Though the E2F site mutated in the T2P Mut. B is not conserved (figure 5.8 (highlighted in blue)), a second E2F binding site was identified in the human TRIB2 promoter (figure 5.8 (highlighted in red)). These two human E2F sites lie within 150bp of each other in the TRIB2 promoter. A primer set that flanks both the human E2F sites was designed along with a control set of primers that flanked a region of DNA -5kb upstream of the TRIB2 transcriptional start site. Chip analyses revealed that this region of the TRIB2 promoter is enriched for binding of E2F1, E2F2 and E2F3 but not E2F4 and E2F5 (figure 5.10 (a)). Analyses of the pull down of E2F1, E2F2, E2F3, E2F4 and E2F5 proteins using their relevant antibodies in the Chip system indicated that all Immunoprecipitations (IPs) were successful (figure 5.10 (b).

Figure 5.8: ClustalW2 of the human (Homo_sapiens), murine (Mus_musculus) and Orangutan (Pongo_pygmaeus_abelii) TRIB2 5'-UTR. Murine E2F binding site B is coloured in blue and site C is coloured in pink. Site in red is an E2F binding site identified in the human TRIB2 promoter.

Factor	Model	Beg	Sns	Len	Sequence	L_a	$L_{a/}$	Lq	Ld
E2F + P107	R08844 ()	-804	N	6	TCGCGG	12	2	1	0
E2F	R01851 ()	-1036	N	8	GCGGGAAA	16	2	1	0

Table 5.II: E2F binding sites in the human TRIB2 promoter comparable to the E2F binding sites B and C identified in the murine TRIB2 promoter by analysis using TESS. Model is the site string or weight matrix used to pick the site, Beg gives the location of the start of the site in the sequence (0 is the transcriptional start site for TRIB2), Sns is the sense of the site (N is normal, R is reverse compliment), Len is the length of the site, Sequence is the sequence of the binding site matched to the model, La is the Log-likelihood score (the higher the better), La/ is the La /Len (higher is better, maximum value is 2.00), Lq is the La/L_M (L_M is the maximum La possible for the site model, higher is better and the maximum values if 1.00) and Ld is the L_M – La (0 is the best value, higher values indicate a sores match).

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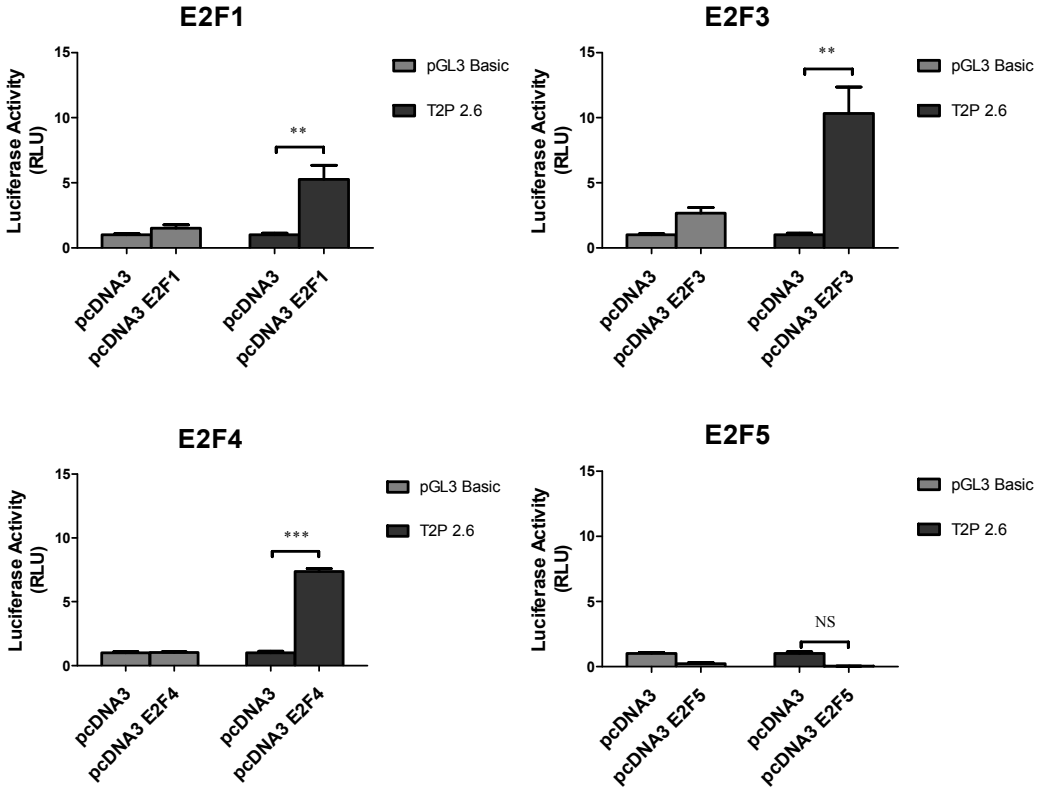
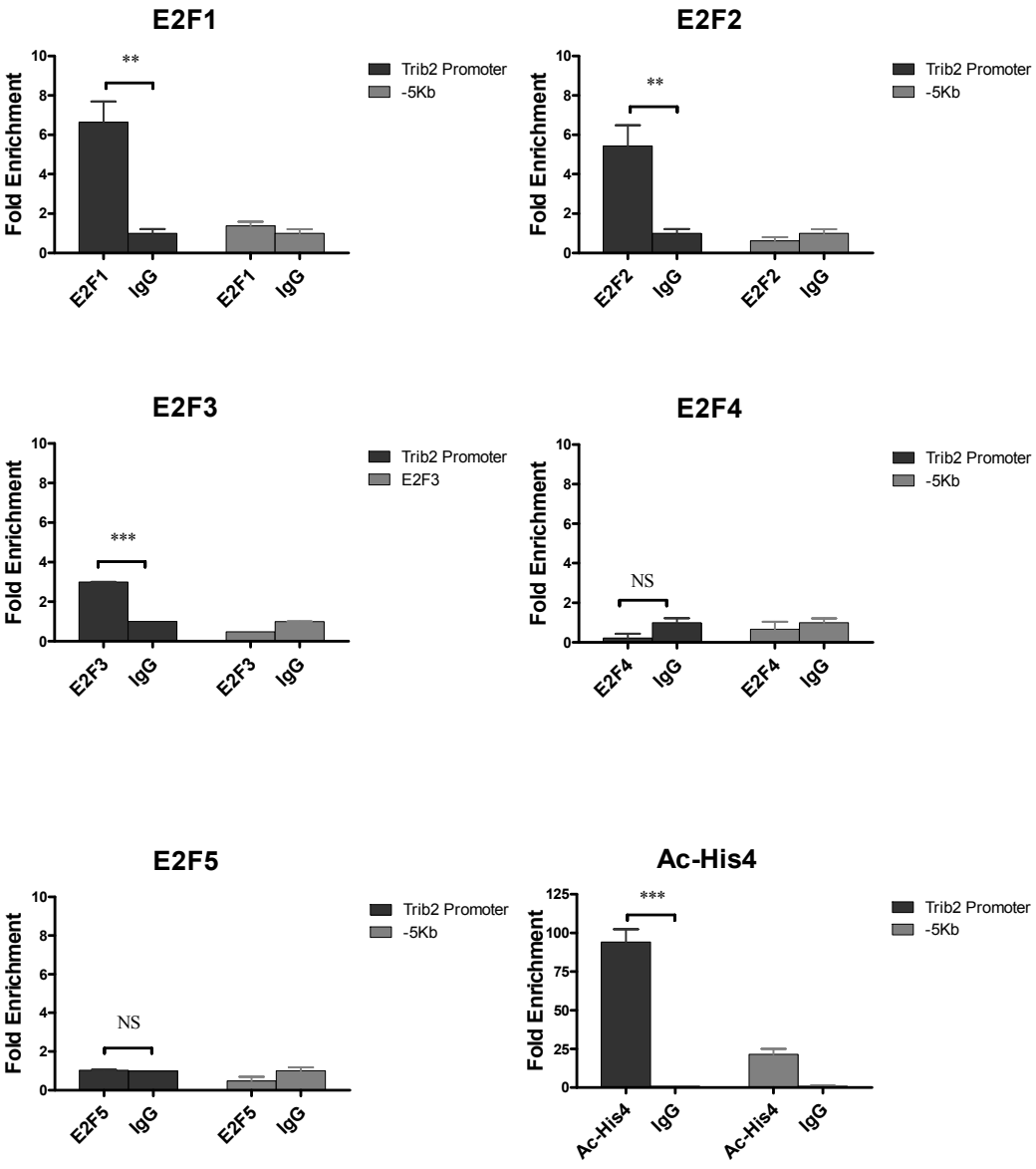


Figure 5.9: Other members of the E2F transcription factor family can activate the T2P 2.6 and E2F1, 2 and 3 all bind the Trib2 promoter region. E2F1, 3 and 4 can all activate the T2P 2.6. Values are normalised to relevant promoter transfected with control (pcDNA3) and are representative of experiments performed in duplicate. Error bars indicate +/- SD of duplicate samples. Significance was calculated by two-way ANOVA analyses with bonferroni post test. A p-value below 0.05 is considered significant, p-value below 0.05 is represented by *, below 0.01 by ** and below 0.001 by ***.

a)


E2F1
Fold Enrichment
10
8
6
4
2
0
**
E2F1
IgG
E2F1
IgG
Trib2 Promoter
-5Kb
E2F2
Fold Enrichment
**
E2F2
IgG
E2F2
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Trib2 Promoter
-5Kb
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E2F3
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IgG
Trib2 Promoter
-5Kb
E2F5
Fold Enrichment
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IgG
E2F5
IgG
Trib2 Promoter
-5Kb
Ac-His4
Fold Enrichment
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Trib2 Promoter
-5Kb

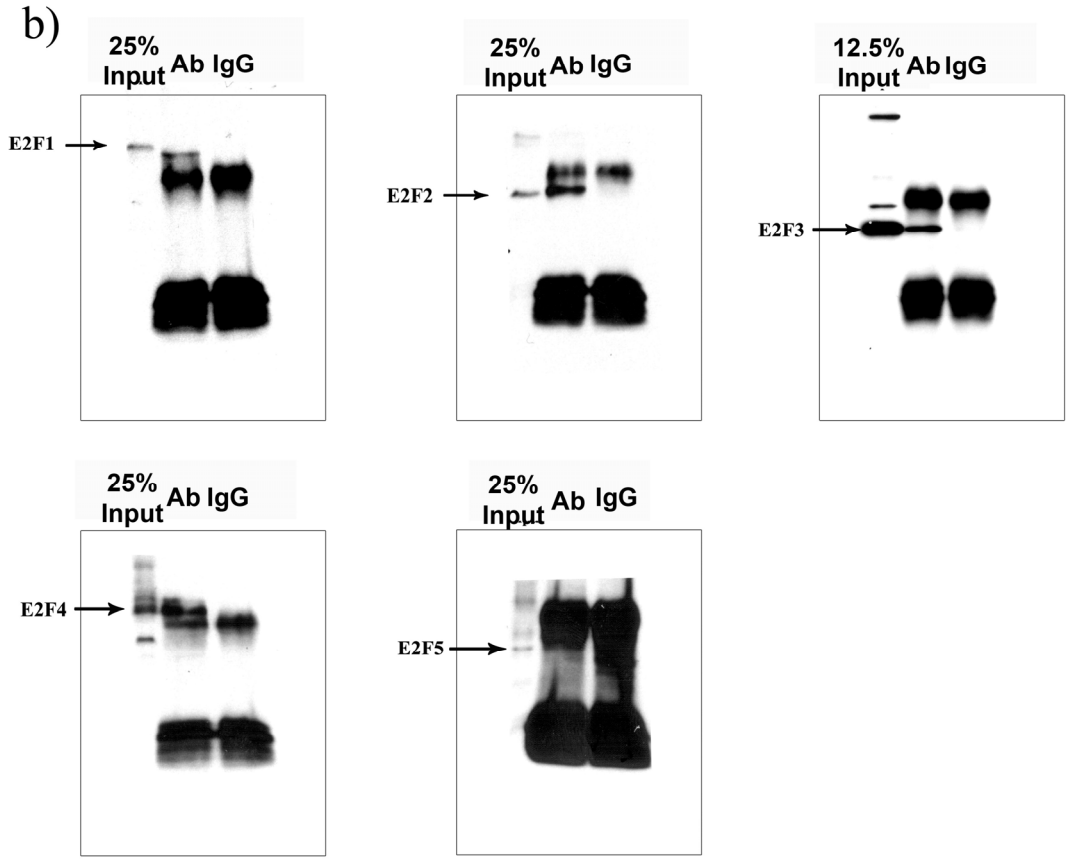

Figure 5.10: E2F1, 2 and 3 all bind the human TRIB2 promoter region that contains the E2F binding sites illustrated in the schematic. DNA from K562 cells pulled down by the E2F1, 2, 3, 4 and 5 proteins was analysed by real-time PCR for presence of the TRIB2 promoter region indicated in **a)** and for a control region of DNA that does contain any E2F binding sites -5Kb upstream of the transcriptional start site of TRIB2. Presence of DNA from these two regions was compared to pull-down with a control (Rabbit IgG). Values are representative of experiments performed in triplicate. Error bars indicate +/- SD of duplicate samples. Significance was calculated by two-way ANOVA analyses with bonferroni post test. A p-value below 0.05 is considered significant, p-value below 0.05 is represented by *, below 0.01 by ** and below 0.001 by ***. **b)** Western blots of successful pulldown of E2F1, 2, 3, 4 and 5 by their relative antibodies in K562 cells.

5.5 E2F1 induces the expression of TRIB2 mRNA and protein

The luciferase (figures 5.4 and 5.9) and chip assays (figure 5.10 (a)) indicate that E2F1 is binding to and activating the TRIB2 promoter. In order to demonstrate that E2F1 induced TRIB2 promoter activity can affect TRIB2 expression levels in the cell mRNA in both 293T and E2F1 Knockout (KO) MEF cells and protein levels in the E2F1 KO MEF cells of TRIB2 were analysed post E2F1 transfection. Intracellular Notch1 (ICNX), a constitutively activated form of Notch was used as a positive control for these experiments as TRIB2 has been shown to be a Notch1 target (Wouters et al., 2007).

mRNA expression was measured by RT-PCR and results show that there was a significant increase in Trib2 mRNA expression 24 hours post transfection of the E2F1 KO MEF with E2F1 (figure 5.11 (a)). The same increase can be seen in 293T cells 48hrs post transfection (figure 5.11 (a)). Analyses of the protein levels by western blotting using a TRIB2 antibody showed that Trib2 protein expression in the E2F1 KO MEF was significantly lower compared to wild-type MEFs (figure 5.11 (b)). Transfection of E2F1 into the E2F1 KO MEFs resulted in a significant increase in Trib2 expression compared with the transfection of the empty vector pcDNA3 24 and 48 hours post transfection (figure 5.11 (c)). A significant increase in Trib2 expression post-transfection with ICNX was also seen after 48 hours (figure 5.11 (c)). Densitometric analyses of Trib2 expression 48 hours post-transfection of pcDNA3 (control), E2F1, ICNX or Trib2 demonstrated that there is a significant increase in Trib2 expression post E2F1 transfection (figure 5.11 (d)) as well as post ICNX (positive control) and Trib2 transfection (figure 5.11 (d)).

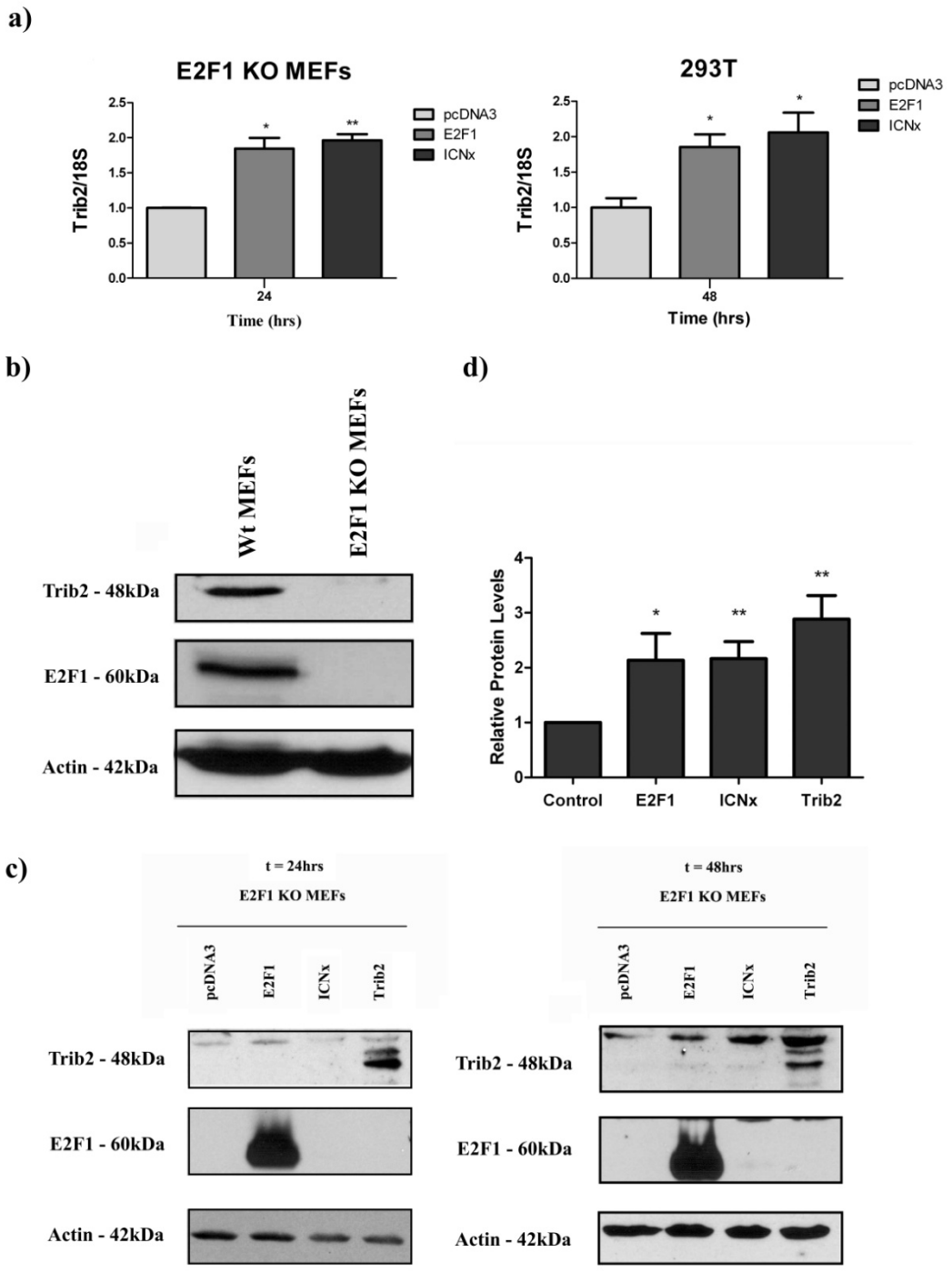

Figure 5.11: Over-expression of E2F1 can induce TRIB2 mRNA and protein in the cell. **a)** Transfection of E2F1 into both 293T cells (48 hours post-transfection) and E2F1 KO MEFs (24 hours post-transfection) leads to an increase in TRIB2 mRNA expression. A similar increase in TRIB2 mRNA levels is seen after transfection of ICNx (positive control). TRIB2 mRNA levels were analysed by real-time PCR, values are representative of experiments performed in duplicate and are normalised to transfection with control (pcDNA3). Error bars indicate +/- SD of duplicate samples. Increase in TRIB2 expression levels were compared to control (transfection with pcDNA3), significance was determined using the student t-test. A p-value

below 0.05 is considered significant, p-value below 0.05 is represented by *, below 0.01 by ** and below 0.001 by ***. **b)** Western blot analyses of wt and E2F1 KO MEFs shows that Trib2 protein levels are higher in the wt cells. 150ug of protein isolated from wt and E2F1 KO cells using Tris buffer were run on a 10% SDS-gel. Results are representative of experiments performed in duplicate. **c)** Transfection of E2F1 KO MEFs with E2F1 (and ICNx, positive control) leads to an increase in Trib2 protein levels 24 and 48 hours post transfection compared to control (pcDNA3). Samples were run on a 10% SDS gel and were probed for Trib2, E2F1 and Actin which acts as a loading control. 80µg of protein were loaded into each well. Results are representative of experiments performed in triplicate. **d)** Densitometric analyses of Trib2 levels in E2F1 KO MEFs 48 hours post pcDNA3, E2F1, ICNx or Trib2 transfection. Values are the average of experiments performed in triplicate. Increase in Trib2 expression levels were compared to control (transfection with pcDNA3), significance was determined using the student t-test. A p-value below 0.05 is considered significant, p-value below 0.05 is represented by *, below 0.01 by ** and below 0.001 by ***.

5.6 C/EBPα Represses E2F1 activation the T2P 2.6 reporter

Gilby et al., 2010 reported that Trib2 expression can be induced by the C/EBPα myeloid transcription factor; however we found that the C/EBPα transcription factor significantly negatively regulates the activation of the T2P 2.6 reporter in these studies (figure 5.4). TESS analysis identified a number of putative C/EBPalpha binding sites in the Trib2 promoter (table 5.I). A number of these sites are located within the E2F regulatory region of the Trib2 promoter that we have previously identified (figure 5. (d and e)). As C/EBPα mediated repression of E2F1 activity is crucial for myeloid differentiation (Rosenbauer and Tenen, 2007) we wished to determine if an E2F1-C/EBPalpha feedback loop was involved in the regulating Trib2 expression. Co-transfection of the E2F1 and the p42 form of the C/EBPα transcription factor in 3T3 cells lead to a significant decrease in E2F1 activation of the T2P 2.6 reporter compared to transfection with E2F1 alone (figure 5.12 (a)).

C/EBPα is often found mutated in AML (Nerlov, 2004). These mutations often lead to the abolition of the expression of the full form of C/EBPα, the C/EBPα-p42 isoform, and instead increase in the expression of a truncated form of C/EBPα, C/EBPα-p30 (Nerlov, 2004). Mice that express the p30 isoform of C/EBPα, but are deficient in the expression of the p42 form of C/EBPα, develop AML with complete penetrance (Kirstetter et al., 2008). In comparison C/EBPα KO mice do not develop AML (Zhang et al., 1997; Heath et al., 2004; Zhang et al., 2004) indicating that it the dysregulated C/EBPα signal and not the absence of C/EBPα that results in leukaemogenesis.

As dysregulated C/EBPα expression leads to AML we wished to determine if Trib2 is a target of the p30 isoform of C/EBPα. It has been previously shown that the p30

isoform of C/EBPα can recruit E2F1 transcription factor to the promoter region of PIN1 and by doing so increases the activation of the promoter region of PIN1 compared to E2F1 alone (Pulikkan et al., 2010) suggesting that the p30 isoform of C/EBPα may cooperate with E2F1 to activate E2F1 target genes. Indeed co-transfection of E2F1 and the p30 isoform of the C/EBPα transcription factor resulted in a significant increase in E2F1 activation of the T2P 2.6 reporter compared to transfection with E2F1 alone (figure 5.12 (b)).

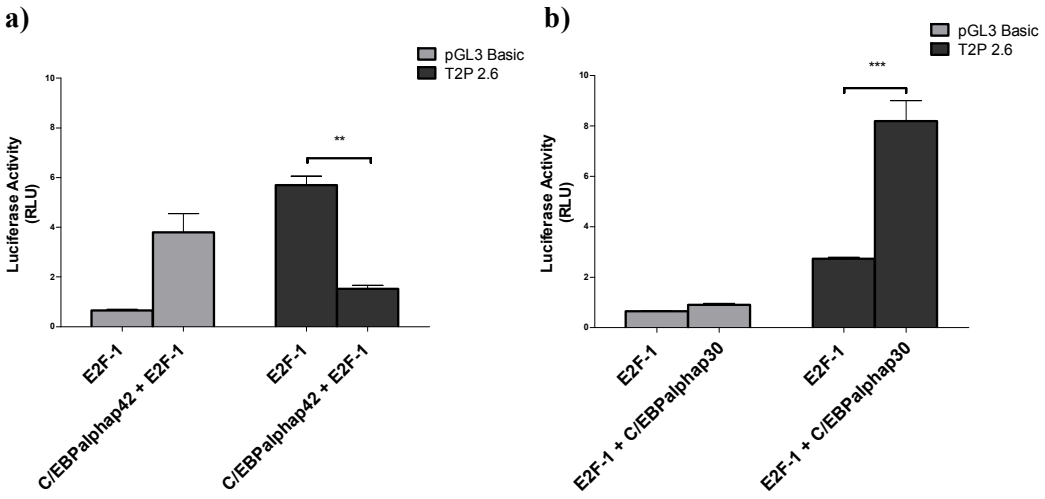

Figure 5.12: Induction of C/EBPα p42 or C/EBPα p30 led to no significant change in Trib2 expression levels in the cell. **a)** Co-transfection of C/EBPα-p42 and E2F1 leads to a decrease in the activation of the T2P 2.6 promoter construct compared to transfection with E2F1 alone. Values are normalised to relevant promoter transfected with control (pcDNA3) and are representative of experiments performed in duplicate. Error bars indicate +/- SD of duplicate samples. Significance was calculated by two-way ANOVA analyses with bonferroni post test. A p-value below 0.05 is considered significant, p-value below 0.05 is represented by *, below 0.01 by ** and below 0.001 by ***. **b)** Co-transfection of C/EBPα-p30 and E2F1 leads to an increase in the activation of the T2P 2.6 promoter construct compared to transfection with E2F1 alone Values are normalised to relevant promoter transfected with control (pcDNA3) and are representative of experiments performed in duplicate. Error bars indicate +/- SD of duplicate samples. Significance was calculated by two-way ANOVA analyses with bonferroni post test. A p-value below 0.05 is considered significant, p-value below 0.05 is represented by *, below 0.01 by ** and below 0.001 by ***.

This data suggests a regulatory model by which Trib2 expression is regulated in the cell. E2F1 potentially activates Trib2 expression and can do so in cooperation with C/EBPα p30 (figure 5.13). A negative regulatory loop exits between E2F1 and C/EBPα p42 and C/EBPα p42 may negatively regulate E2F1 activation of Trib2 expression (figure 5.13). Finally the Trib2 protein, which is able to degrade the p42, but not the p30, isoform of the C/EBPα protein may prevent C/EBPα p42 repression of its activity (figure 5.13).

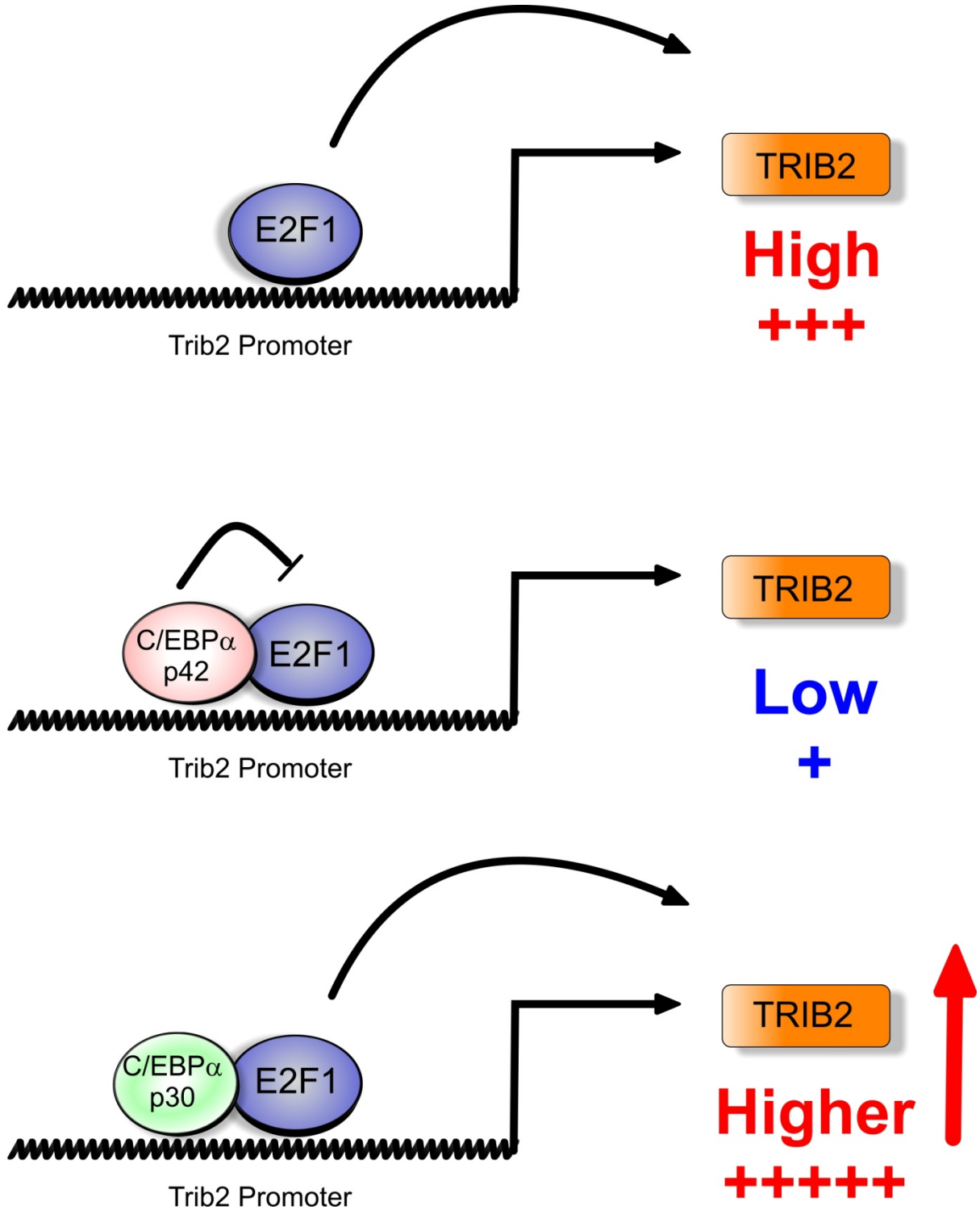

Figure 5.13: Schematic of the model of the regulation of theTRIB2 promoter. E2F1 binds to the TRIB2 promoter activating TRIB2 expression. Binding of C/EBPα p42 to E2F1 represses E2F1 activation of the TRIB2 promoter. However binding of C/EBPα p30 and E2F1 leads to and increased activation of the TRIB2 promoter.

5.7 TRIB2 expression Correlates with E2F expression in AML

As discussed in chapter 4 (results section 4.9) targets of the E2F transcription factors enriches for the TRIB2 signature in the ALL with t(1;19) patients samples, in NK cells (figure 4.11(a and b)) and in the monocytic lineage (figure 4.11(a and c)). Together with the data presented in this chapter these findings indicate that TRIB2 is a target of E2F transcription factors putatively in both normal haematopoiesis and leukaemogenesis.

Further investigation of TRIB2 expression in AML indicates that TRIB2 expression correlates with that of the E2F1 and E2F3 members of the E2F transcription factor family in the AML samples with high TRIB2 expression levels. Analysis of the Valk dataset, a previously published dataset of AML patient samples, revealed that TRIB2 expression positively correlates with that of E2F1 and E2F3 in the high TRIB2 expressing samples (figure 5.14). No statistically significant correlation between TRIB2 expression and the other members of the E2F transcription factor family was observed. This data suggests that E2F family members may play a role in the regulation of TRIB2 expression in AML, particularly E2F1 and E2F3 which positively correlate with TRIB2 expression.

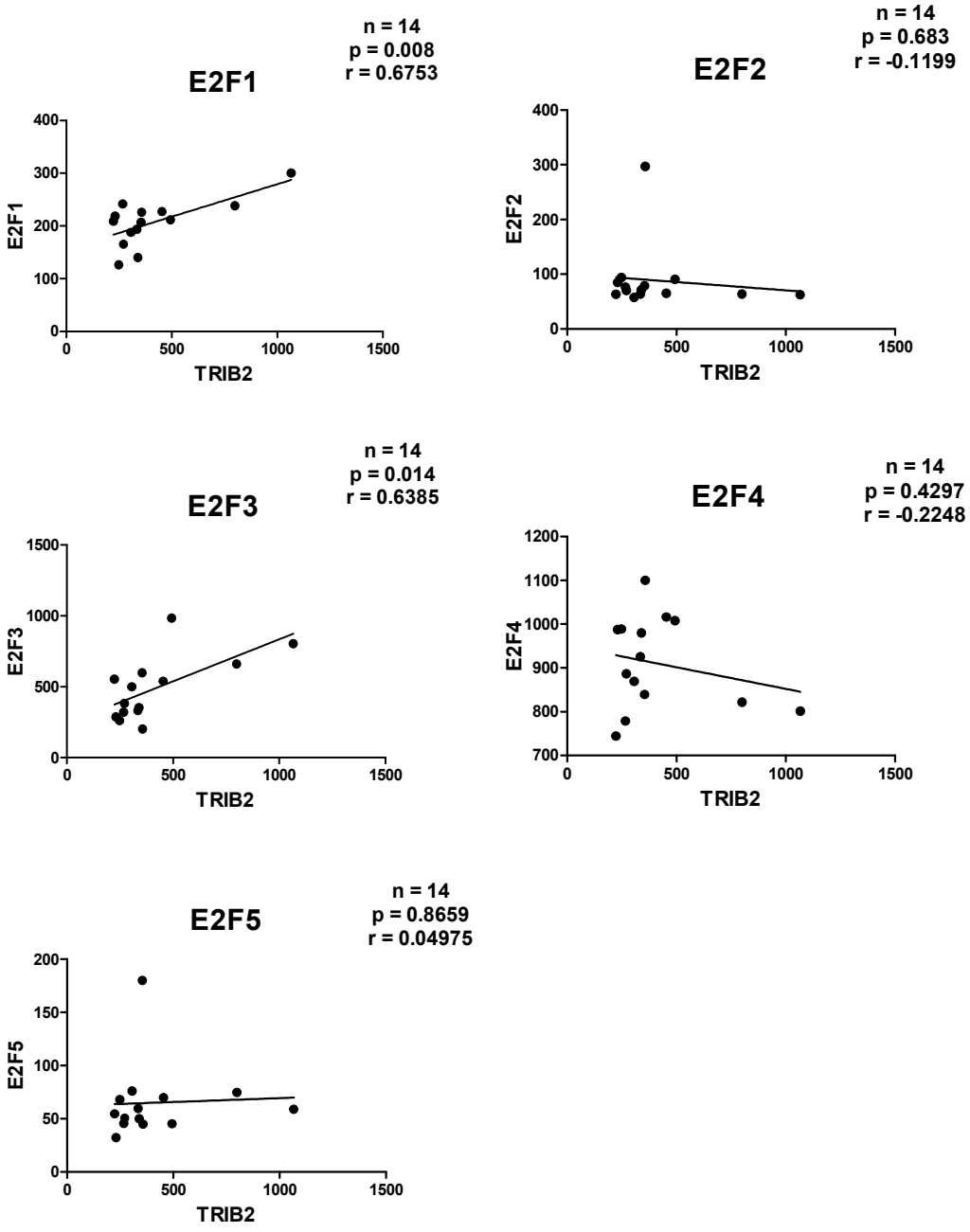

Figure 5.14: TRIB2 expression correlates with both E2F1 and 3 expression in AML. Correlation analysis of TRIB2 versus E2F1, 2, 3, 4, and 5 in the top 95 percentile of patient samples (n=14) based on TRIB2 expression from the Valk dataset (GSE1159)(Valk et al., 2004). p-value (<0.05) and correlation coefficient (Pearson's r, close to +1) indicate significant positive correlations. Values on the x and y axis indicate relative expression values for these genes derived from the Valk microarray data.

Discussion

Understanding the regulation of TRIB2, both in normal and in leukemic cells, is important as it will help identify potential therapeutic targets for leukemic patients with high TRIB2 signatures. Here it has been determined that there is a direct regulatory link between E2F1 and TRIB2 expression in the cell. Analyses of TRIB2 expression in the leukemic subsets of the MILE study, in the normal cells of haematopoiesis, and in a number of leukemic cell lines revealed that TRIB2 expression is dynamic and varies depending on cell type. Further investigation into transcription factors that may be controlling the expression of TRIB2 identified the E2F family of transcription factors as possible regulators of TRIB2 expression. The TRIB2 promoter region was shown to contain a number of potential E2F binding sites within 2.6Kb of the transcriptional start site of TRIB2. Luciferase assays using the TRIB2 promoter construct revealed that E2F1 can activate the TRIB2 promoter. Having narrowed down the region of the TRIB2 promoter to which E2F1 can bind a number of chromatin immunoprecipitation assays demonstrated that not only E2F1 but E2F2 and E2F3 bind the TRIB2 promoter but E2F2 and E2F3 as well. Expression levels of TRIB2 in E2F1 KO MEFs proved to be lower than that of wt MEFs. Over-expression of E2F1 in both 293T cells and E2F1 knock out MEFs led to an increase in TRIB2 RNA levels. Over-expression of E2F1 also led to an increase in TRIB2 protein levels in E2F1 KO MEFs.

An important and necessary step in granulocytic differentiation is the repression of E2F dependent transcription by the C/EBPα myeloid transcription factor (Rosenbauer and Tenen, 2007). The full form C/EBPα-p42 isoform, is able to repress the E2F1-induced activation of TRIB2 promoter whereas the truncated form,

C/EBPα-p30, can increase activation of the TRIB2 promoter compared to E2F1 alone. This suggests that C/EBPα and E2F1 may be involved in a regulatory loop that controls TRIB2 expression during myeloid differentiation.

Both TRIB2 and the E2F transcription factor family are associated with pathways in survival, proliferation and differentiation. TRIB2 expression is dynamically controlled during haematopoiesis and that it is a direct target of E2F1 in the cell (chapter 5). As mice deficient in E2F1, E2F2, both E2F1 and E2F2 or E2F1 E2F2 and E2F3 all show defects in haematopoiesis (Matsumoto and Nakayama, 2013) this suggests that E2F1 and perhaps other members of the E2F transcription factor family may play an important role in the regulation of TRIB2 expression during haematopoietic cellular differentiation.

Chapter 6

General Discussion

6.1 Summary and Overall Conclusions

The Trib1 and Trib2 genes of the Tribble gene family were first identified as leukaemia causing genes (Jin et al., 2007; Keeshan et al., 2006). Since then all three members of the Tribbles family have been associated with cancer (Grandinetti et al., 2011; Miyoshi et al., 2009; Puiffe et al., 2007; Wennemers et al., 2011a, 2011b; Zanella et al., 2010; Zhang et al., 2012).

Profiling of the expression of the Tribbles genes was performed in order to gain some insight into the role of the Tribbles genes in haematopoiesis and identify leukaemic subtypes in which they might play a role. Profiling of the Tribbles genes TRIB1 and TRIB2, the two genes most closely related to leukaemogenesis, illustrated the fact that these two genes posses distinct expression profiles across the haematopoietic cellular compartments. TRIB1 expression was highest in the monocytic cellular compartment and TRIB1 expression was significantly increased in the terminally differentiated monocytic cells indicating that TRIB1 expression is induced during monopoiesis and remains high in the terminally differentiated cells. Significant differences in TRIB1 expression were also observed during B cell development and between the NK cell and the dentritic cell subtypes.

In contrast to TRIB1, TRIB2 expression was highest in the lymphoid compartments of haematopoiesis. Of all the cells of the haematopoietic system TRIB2 is most highly expressed in the T cell compartment and is also high in the B cell and NK cellular compartments. Breakdown of TRIB2 expression within the haematopoietic lineages revealed that TRIB2 expression is significantly higher in the CD8+ T cells compared to the CD4+ cells and that TRIB2 expression significantly increases during B cell development and significantly decreases during erythroid cellular

differentiation. Like TRIB1, TRIB2 expression is significantly higher in the myeloid compared to the plasmacytoid dendritic cells and shows significant differences in expression between the different NK cell types. Interestingly, TRIB2 expression is increased in the NK cell types that posses decreased TRIB1 expression. Differences in TRIB2 expression between the different cellular subtypes of specific compartments (NK and T Cell) suggest that TRIB2 expression levels may have a functional role in these cells. The variation in TRIB2 expression as certain cellular populations differentiate (e.g. B cell and erythroid) also indicates that dynamic regulation of TRIB2 expression may be an important part of cellular maturation.

TRIB2 expression is also dynamically controlled during myeloid cell differentiation. As previously discussed TRIB2 expression decreases during erythroid development, a decrease in TRIB2 expression is also observed in monopoiesis and proved to be lowest in the monocytes compared to all other cellular compartments of haematopoiesis. TRIB2 expression is significantly increased in the terminal populations of Granulocytes, Basophils, Eosinophils and Megakaryocytes compared to the earlier HSC and CMP populations. While TRIB1 and TRIB2 expression can follow similar profiles, for example in the dendritic cells and during granulopoiesis, often their expression is controlled in opposing directions, for example during monopoiesis and in the NK cell subpopulations, suggesting that there may be redundancy between these Tribbles genes during haematopoiesis.

TRIB3 expression was not dynamically controlled during haematopoiesis and there were no significant differences in TRIB3 expression between the different cellular compartments. However TRIB3 expression proved to be significantly higher in the granulocyte compartment compared the monocytes, basophil and eosinophil compartments only. Of the three Tribbles genes, Trib3 does not induce murine AML

(Dedhia et al., 2010) and here we see that TRIB3 expression is not dynamically controlled indicating that regulation of TRIB3 is not be an important facet of haematopoiesis.

Clustering of the TRIB1, 2 and 3 signatures based on the expression of these genes reflected the findings of the profiles of these three genes in haematopoiesis. The high TRIB1 signature clusters within the monocytic and granulocytic compartments and the low TRIB1 signature clusters within the T cell compartment. In contrast the high TRIB2 signature T cell along with the B cell and NKA cell samples. This further associated TRIB1 expression with the monocyte/myeloid compartment while TRIB2 expression is most closely associated with the lymphoid compartment. Finally TRIB3, whose expression is not dynamically controlled during haematopoiesis, fails to cluster with any specific cell type.

Having associated TRIB1 and TRIB2 expression with specific haematopoiesis cellular compartments and identified their dynamic expression during haematopoiesis, further analysis of Tribbles expression was performed in leukaemic cells to investigate the role of these genes in leukaemogenesis. Analyses of each of the Tribble levels in the various leukaemic subtypes of the MILE study revealed that TRIB2 expression is associated with the ALL leukaemic subtypes in particular the T-ALL and ALL with t(1;19) subtypes which both had increased TRIB2 expression compared to the control group and the other leukaemic subtypes. Lymphoid leukaemia arises from mutated lymphoid cells, the cellular compartment which we found was most closely related to TRIB2 expression. Clustering of TRIB2 expression within ALL revealed that the high TRIB2 signature clustered with the T-ALL and ALL with t(1;19) subtypes further associating TRIB2 expression with these leukaemic subtypes.

TRIB2 was first identified as a myeloid leukaemic gene (Keeshan et al., 2006) and although our analysis indicated that aberrant TRIB2 expression was associated with ALL we discovered a subset of AML with normal karyotype and other abnormalities that possessed increased TRIB2 expression compared to the control group.

Surprisingly TRIB1 analyses revealed that none of the leukaemic subtypes expressed significantly higher TRIB1 expression when compared to control group samples, although overall TRIB1 expression was higher in the AML subtypes compared to the ALL subtypes. Myeloid leukaemia arises from mutated myeloid cells and this is the compartment most closely related to TRIB1 expression. Despite the fact that none of the specific subtypes showed significant increased TRIB1 expression compared to the control group aberrant TRIB1 has also been described in a patient with AML (Röthlisberger et al., 2007) and TRIB1 is a myeloid leukaemia causing gene (Dedhia et al., 2010; Jin et al., 2007). However comparison of TRIB1 levels with the total bone marrow samples of the control group may be masking an increase in TRIB1 and a more direct comparison of TRIB1 levels in the leukaemic cells with those of the HSC or myeloid progenitor cell may reveal an aberrant increase in TRIB1 expression.

Clustering of TRIB1 expression within the AML and ALL subtypes revealed that no specific cluster of AML or ALL patients were associated with TRIB1 expression. However a subset of AML with normal karyotype and other abnormalities did cluster with a high TRIB1 signature indicating that a group of these samples does posses a TRIB1 signature that may be associated with the development of leukaemia.

GSEA analysis of the TRIB1 and TRIB2 signatures in the various subsets of leukaemia reinforces the finding that TRIB1 expression is associated with myeloid gene expression and TRIB2 is associated with lymphoid gene expression.

TRIB3 expression was found to be significantly higher in a number of leukaemic subtypes when compared to the control group however the evidence for TRIB3 involvement in leukaemia is limited. The TRIB3 signature did cluster T-ALL and Mature B-ALL with t(8;14) samples in ALL and a mixture of AML with normal karyotype and other abnormalities and AML with complex aberrant karyotype in AML. Despite this TRIB3 expression is not dynamically controlled in during haematopoiesis and increased TRIB3 expression does not induce leukaemia (Dedhia et al., 2010). There is little evidence that TRIB3 expression itself causes leukaemia but TRIB3 is induced by the stress response pathway (Koh et al., 2013) and increased TRIB3 expression may be as a result of activation of this response in leukaemic cells. TRIB3 expression has been linked to prognosis in colon and breast cancer (Miyoshi et al., 2009; Wennemers et al., 2011a, 2011b) and a role in leukaemic cell survival cannot be ruled out

Using the TRIB1, 2 and 3 signatures in the various subtypes of leukaemia found in the MILE study, connectivity mapping analyses identified a number of drug candidates linked to these gene signatures. A number of drugs were identified that may be able to reverse a high TRIB1, 2 or 3 signature in the cell thereby treating leukaemia with these profiles. Drugs that may induce both the TRIB1 and TRIB2 signatures include the HDAC inhibitors indicating that this drug connects with both these signatures and have been recently shown to induce TRIB1 expression in the cell (Liss et al., 2013). Halofantrine is an antimalarial drug believed to inhibit autophagy negatively connects with the TRIB2 signature in the ALL disease state

possibly linking TRIB2 expression and this cellular function. The PI3K LY-294002 positively connects with TRIB2 in AML and negatively connects with the TRIB3 signature both in the AML, CLL and CML disease states. This may indicate opposing functions of these two Tribbles genes and links both to the PI3K/AKT signalling pathway, a pathway which both have been shown to regulate (Naiki et al., 2007; C. Zhang et al., 2011).

As both Trib1 and Trib2 induce murine leukaemia and are dynamically controlled in haematopoiesis further analysis of these two genes was performed to characterise their function in both the normal haematopoietic and leukaemic cell. Identification of the pathways involving TRIB1 and TRIB2 and potential transcription factors involved in the regulation of these two genes is an important step in the investigation into what roles these two genes might play in haematopoiesis in the development of leukaemia. Analyses of the TRIB1 and TRIB2 signatures identified a number of cellular pathways and transcription factor families that may be involved in the regulation of either TRIB1 or TRIB2 expression.

The pathways associated with both TRIB1 and TRIB2 expression proved to be highly consistent with previously published data. This may be reflective of the high quality data produced by the MILE study. Unlike other leukaemia sample sets, which are generally produced from relatively small, single-centre studies involving archival samples, all samples analysed for the MILE study were obtained from untreated patients at the time of diagnosis. The operators at each centre were trained to use the same sample preparation protocol and were provided with identical laboratory equipment along with kits and reagents from the same source for sample preparation and analysis. Prephase testing of samples from each of the centres showed that the analysis results were highly reproducible and accurate (Haferlach et al., 2010;

Kohlmann et al., 2008). Accurate sub classification of the different types of leukaemias by a number of methods including cytogenetics and immunophenotyping also allows for the production of consistent data, something that is not enjoyed by other areas. All this has allowed for the production of a number of high quality leads in the investigation of the roles played by TRIB1 and TRIB2 in leukaemia.

Pathways enriched for the TRIB1 signature specifically in the AML samples with normal karyotype included TLR pathways, G-protein coupled receptor pathways, Wnt/β-catenin Signalling Pathway and NF-κB signalling pathways. A number of these pathways have been previously associated with TRIB1 expression including the NF-κB pathway as TRIB1 is known to act as a co-activator for RelA, a subunit of NF-κB, promoting the induction of proinflammatory cytokines in adipocytes (Ostertag et al., 2010). Selective enrichment of pathways, such as the Notch signalling pathway for the TRIB1 signature only in the monocyte lineage, indicated specific association of the TRIB1 signature with certain signalling pathways during development of specific cellular lineages and showed a difference in TRIB1 signalling between the normal haematopoietic and leukaemic cell. Enrichment of pathways such as NF-κB pathways, TLR pathways and immune system signalling pathways for the TRIB1 signature in both normal haematopoietic and leukaemic cells indicated that the TRIB1 signature also associated with similar pathways in both states. Dysregulated TRIB1 function may aberrantly affect these pathways leading to mutation of the cell. For example increased MAPK signalling has already been identified as a hallmark of TRIB1induced AML (Jin et al., 2007; Yokoyama et al., 2010) and this pathway was enriched for the TRIB1 signature in many of the leukaemic subtypes and haematopoietic compartments and lineages.

TRIB2 proved to be enriched for T cell and T cell Co-Stimulation Pathways, TLR pathways, apoptosis associated pathways, B cell pathways and immune system signalling pathways across the T-ALL, ALL with t(1;19) and AML with normal karyotype patient samples, all leukaemic subtypes associated with aberrant TRIB2 expression. Enrichment of T cell and B cell associated pathways further supports the association of TRIB2 expression with the lymphoid compartment. Enrichment of apoptosis associated pathways is of interest as TRIB2 has been associated with both pro and anti apoptotic activity in the cell (Gilby et al., 2010; Grandinetti et al., 2011; Lin et al., 2007).

Enrichment of the Notch signalling pathway for TRIB2 in the T-ALL patient samples is particularly of interest as TRIB2 is a Notch1 target (Wouters et al., 2007) and activating Notch1 mutations are a hallmark of T-ALL (Erbilgin et al., 2010)**.** we discovered that TRIB2 expression is increased in paediatric T-ALL patient samples with mutations associated with increase Notch1 activity indicating that TRIB2 is one of the targets of aberrant Notch1 signalling driving T-ALL development (see appendix A).

The Wnt/β-catenin Signalling Pathway is also associated with the TRIB2 signature in leukaemia. TRIB2 was recently reported to be a target of this pathway in liver cancer (J. Wang et al., 2013) signalling the possibility that this pathway is also involved in the induction of TRIB2 expression in the leukaemic cell. The TRIB1 signature was also enriched for this pathway in the AML samples with normal karyotype and other abnormalities possible linking the aberrant induction of TRIB1 expression to this pathway in AML. Other identical and similar cellular pathways were also enriched in both the TRIB1 and TRIB2 signatures across the leukaemic subtypes and in the cellular compartments and lineages of normal haematopoiesis.

This indicates that TRIB1 and TRIB2 may play similar roles in the cell, however the vast differences in the level and frequency of enrichment of these pathways shows that TRIB1 and TRIB2 also possess distinctive signatures, they may play both overlapping and unique roles in the cell. This information may lead to the identification of potential therapeutic targets for further study in the treatment of leukaemia.

As with TRIB1 the pathways enriched for the TRIB2 signature in normal haematopoiesis also overlap with those enriched in the leukaemic subtypes. But unique pathways are also enriched for the TRIB1 signature in the haematopoietic lineages such as the Her/ErbB pathway which is enriched in the T cell lineage. This also indicates a specific association between the TRIB2 signature with certain signalling pathways during development of specific cellular lineages and illustrates a difference in TRIB2 signalling between the normal haematopoietic and leukaemic cell.

A number of transcription factor targets were associated with TRIB1 and TRIB2 expression across the leukaemic subtypes and the haematopoietic lineages. Many of these transcription factors have been shown to be involved both in haematopoiesis and in leukaemogenesis adding weight to the idea that TRIB1 and TRIB2 expression is important in haematopoiesis and dysregulation of this expression may lead to leukaemia.

Enrichment for targets of the C/EBP family of transcription factor for the TRIB1 signature was an interesting discovery that suggests that TRIB1 is a target of C/EBP transcription factors in the cell. This was validated by analysis of endogenous TRIB1 response to C/EBPα expression and presents an interesting lead in the identification

of regulators of TRIB1 expression. The data suggests that TRIB1 is a target of the wildtype form of C/EBPα in AML and that TRIB1 expression may be perturbed in AML patient samples with mutated C/EBPα. Low TRIB1 expression has been associated with a group of patient samples with a low C/EBPα signature (Liss et al., 2013). These samples include a number of patients with predominantly biallelic mutations in the C/EBPα gene suggesting that abrogation of wildtype C/EBPα expression in AML may lead to a failure to induce TRIB1 expression. However not all patients with biallelic mutations was associated with this low C/EBPα signature group suggesting that not all mutations to C/EBPα may affect TRIB1 expression.

TFTs of the E2F family of transcriptions factors was one of the families of transcription factors that were identified as possible regulators of TRIB2 both in leukaemia, specifically in the ALL with t(1;19), and in the normal cells of haematopoiesis. Both the natural killer cells and the monocytic cell lineage were found to be enriched for TFTs of the E2F family when analysed using the TRIB2 signature. Increased E2F1 transcriptional activity has been connected with both AML (Pulikkan et al., 2010) and with paediatric T-cell lymphoblastic leukaemia and lymphoma (Bonn et al., 2012), As this transcription factor family is enriched in the leukaemic subtype with the highest TRIB2 expression as well as a cellular compartment (NKA cells) with high TRIB2 expression, it indicated a potential regulatory role for it in the control of TRIB2 expression.

The data presented in this thesis indicated that TRIB2 expression is dependent on cell type and leukaemic subtype. Analyses of the TRIB2 promoter region was shown to contain a number of potential E2F binding sites within 2.6Kb of the transcriptional start site of TRIB2. Luciferase assays using the Trib2 promoter construct revealed that E2F1 can activate the Trib2 promoter. Having narrowed

down the region of the Trib2 promoter to which E2F1 can bind a number of chromatin immunoprecipitation assays demonstrated that not only was E2F1 binding the TRIB2 promoter but E2F2 and E2F3 as well. Expression levels of Trib2 in E2F1 KO MEFs proved to be lower than that of WT MEFs. Over-expression of E2F1 in both 293T cells and KO MEFs lead to an increase in TRIB2 RNA levels. Over-expression of E2F1 also lead to an increase in Trib2 protein levels in E2F1 KO MEFs. Together these data reveal a novel pathway whereby TRIB2 expression is induced by E2F1 in the cell.

A potential regulatory loop mediated by C/EBPα and E2F1 was identified in the control of Trib2 expression. C/EBPα mediated repression of E2F1 activity is crucial for myeloid differentiation (Rosenbauer and Tenen, 2007) and we were able to show that C/EBPα could negatively regulate Trib2 activation. Interestingly the p30 isoform of the C/EBPα protein could cooperate with E2F1 to activate Trib2 expression. As mutations often lead to the abolition of the expression of the full form p42 form of C/EBPα and increase the expression of a truncated p30 form of C/EBPα in leukaemia (Nerlov, 2004) this indicates that this dysregulated state of C/EBPα expression may lead to an increase in TRIB2 expression and leukaemogenesis.

The E2F proteins are regulated primarily through the formation of protein complexes with a number of different proteins. These complexes can negatively regulate E2F activity in the cell. E2F1 has been shown to preferentially bind to the retinoblastoma (pRB) protein, a pocket protein, forming complexes that can include other proteins and which suppress E2F1 activity. Release of E2F1 from the pRB complex through phosphorylation of pRB results in the activation of the E2F1 protein. E2F1 can then bind the DNA resulting in the activation of its target genes. Though all E2Fs can potentially bind to E2F target sites binding of specific binding partners by the E2F

proteins leads to binding site selectivity (Attwooll et al., 2004). HDACs is another protein that has been shown to form complexes with E2F transcription factors and by doing so repress activation of E2F target genes (L. Chen et al., 2012; Emori et al., 2012; Sharma et al., 2013). HDAC inhibitors can promote the recruitment of E2F1 to its target genes activating their expression (Tan et al., 2006; Zhao et al., 2005). As E2F transcription factors enrich for TRIB2 expression in the ALL with t(1;19) leukaemic subtypes and high TRIB2 expression and expression of E2F1 and 3 correlates in AML patient samples. we have identified E2F1 as a transcription factor that is possibly driving aberrant TRIB2 expression in these leukaemic states leading to the transformation of the cell and the development of leukaemia. HDAC inhibitors positively connect with the TRIB2 signature indicating that this small molecule may in fact induce TRIB2 expression in the cell possibly doing so by releasing E2F1 from a repressive protein complex involving HDAC.

The pathways, transcription factors and the potential small molecules presented here have identified putative differential roles of the Trib family members in normal and malignant haematopoiesis. We have identified specific pathways associated with TRIB1 and TRIB2 expression in different haematopoietic lineages as well as the pathways most often enriched for these genes in the disease state providing a potential map for future investigations of the role these genes play in the normal and transformed haematopoietic cell. This will potentially lead to the identification of possible therapeutic targets that may aid in future the treatment of leukaemia.

6.2: Further investigation into pathways and TFTs identified as enriched for the TRIB1 or the TRIB2 signature.

GSEA analyses identified a large number of pathways and TFTs enriched for either the TRIB1 or the TRIB2 signature across the leukaemic subtypes and in the normal cells of haematopoiesis. Some of these pathways and TFTs are unique to specific haematopoietic cells or lineages and/or unique to specific subtypes of leukaemia. Indeed some pathways and TFTs are enriched across a wide range of haematopoietic cells, lineages and leukaemic subtypes.

Like connectivity mapping GSEA is a hypothesis generating tool and results must be backed up by laboratory experimentation in order to draw definitive conclusions. However these data have given us a large insight into the role of TRIB1 and TRIB2 in both normal and leukaemic cell and has proven useful in the identification of transcription factor families that regulate and pathways involving the Tribbles genes. Initial analyses using the information for a number of pathways and TFTs identified the C/EBP family of transcription factors as transcription factors that may regulate TRIB1 expression. Induction of C/EBPα expression in the leukaemic K562 cells showed an increase in TRIB1 mRNA expression proving that TRIB1 is a target of C/EBPα. Serum stimulation of U937 cells also resulted in an increase in TRIB1 expression. The regulation of TRIB1 expression by the serum response pathway in leukaemic cells was also identified as a possible pathway involving TRIB1 expression. As both these hypotheses were confirmed via wet lab experimentation performed by us further bolsters the reliability of the bioinformatic data and shows that the results here are a rich source of information that may be used to direct future research. Further investigation and validation of the pathways and TFTs identified in these analyses is important as it opens up many new potential novel therapeutic

targets for the investigation and treatment of patients that show a high TRIB1 or TRIB2 signature.

6.3: What functional role does E2F activation of TRIB2 expression play in both the normal and leukaemic cell?

GSEA analyses identified the E2F family of transcription factors as potential regulators of TRIB2 expression. Further biological experimentation has shown that E2F1, 3 and 4 can activate the TRIB2 promoter region and that E2F1, E2F2 and E2F3 are physically bound to the TRIB2 promoter. While E2F1 can induce TRIB2 expression in the cell, a functional role for E2F induction of TRIB2 expression has yet to be determined.

E2F1 expression has long been linked to both apoptosis and cellular proliferation as well as, most recently, to autophagy (Engelmann and Pützer, 2012; Polager and Ginsberg, 2008). Induction of TRIB2 expression by E2F1 may be linked to one or more of these pathways as TRIB2 can play both a pro-survival (Zanella et al., 2010; Zhang et al., 2012) or pro-apoptotic role in the cell (Gilby et al., 2010; Keeshan et al., 2010; Lin et al., 2007). GSEA analyses did reveal that the TRIB2 signature is enriched for genes associated with apoptotic pathways as well as pathways such as the EGF signalling pathway which is involved in the promotion of proliferation. Investigation into what functional role the induction of TRIB2 by E2F1 has in the cell will help answer whether E2F1 induces TRIB2 in a pro-survival or pro-apoptotic manner or both in the non-leukaemic and leukaemic cells.

Further investigation into the role the E2F family of transcription factors play in the regulation of Trib2 expression, particularly in a hematopoietic context, could be

investigated using the E2F1 knock out mouse models to provide functional *in vivo* confirmation and context of the regulatory role played by E2F1 regulation of Trib2. Analyses of human leukaemic patient samples with a high TRIB2 expression for expression of E2F transcription factors and other proteins associated with E2F expression will also allow us to establish a link between E2F expression and TRIB2 levels in the leukaemic cell. Finally, using the information that shows that E2F1 can induce TRIB2 expression will allow us to identify prospective therapeutic targets that target this pathway for the potential treatment of leukaemia with high TRIB2 expression.

6.4 Conclusion

The work presented in this thesis provides new insight into the role played by the Tribble genes in both haematopoiesis and in leukaemogenesis. For the first time we have associated the TRIB1 and TRIB2 expression with the myeloid and lymphoid compartments respectively. Pathways and transcription factors associated with TRIB1 and TRIB2 expression in both haematopoietic and leukaemic cell development have been identified. Small molecules associated both positively and negatively with TRIB1, 2 or 3 expression in the different leukaemic types have also been identified. Further experimental work has associated TRIB1 expression with the serum response pathway and C/EBPα expression. For the first time TRIB2 expression has been linked to the E2F transcription factor family and we have shown that TRIB2 is a direct target of E2F1.

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APPENDIX A

APPENDIX B

APPENDIX C

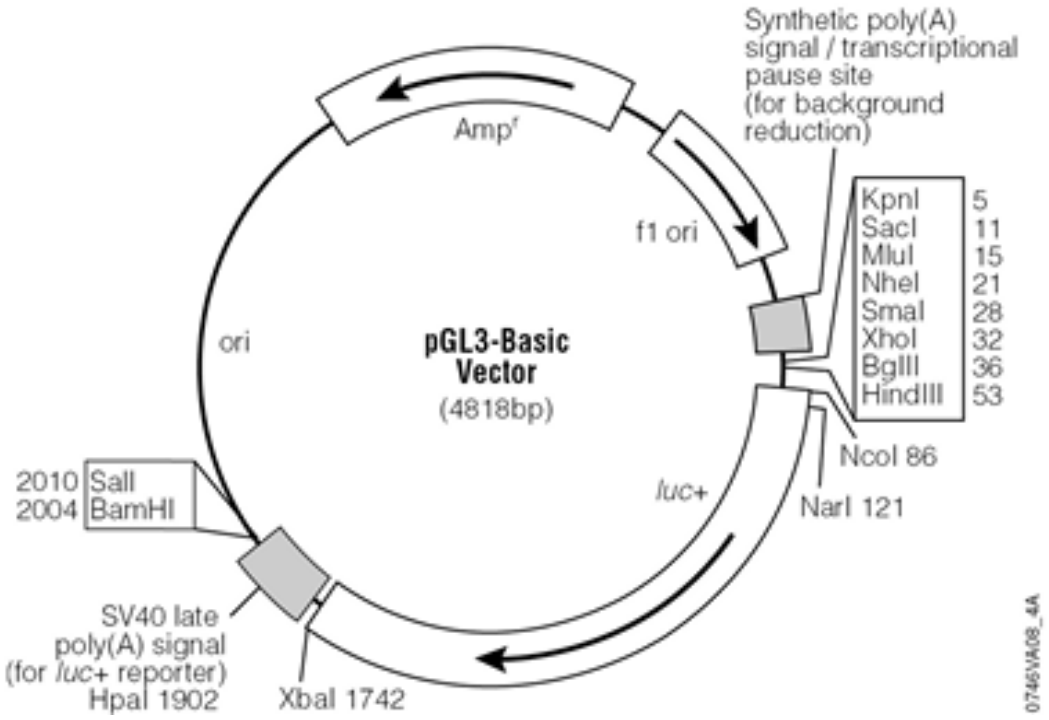


Figure C1: Vector map of pGL3-Basic

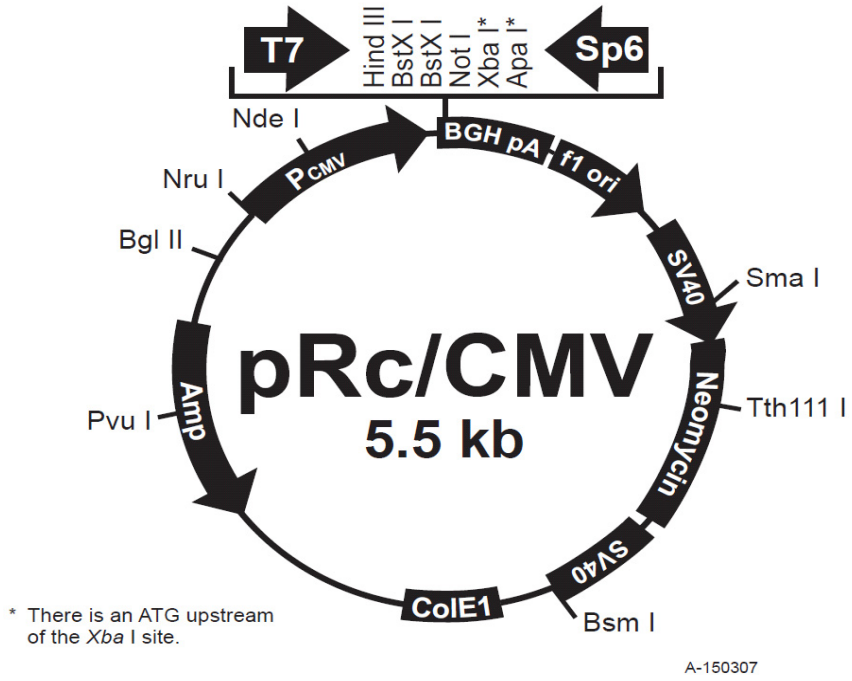


Figure C2: Vector map of pRc/CMV

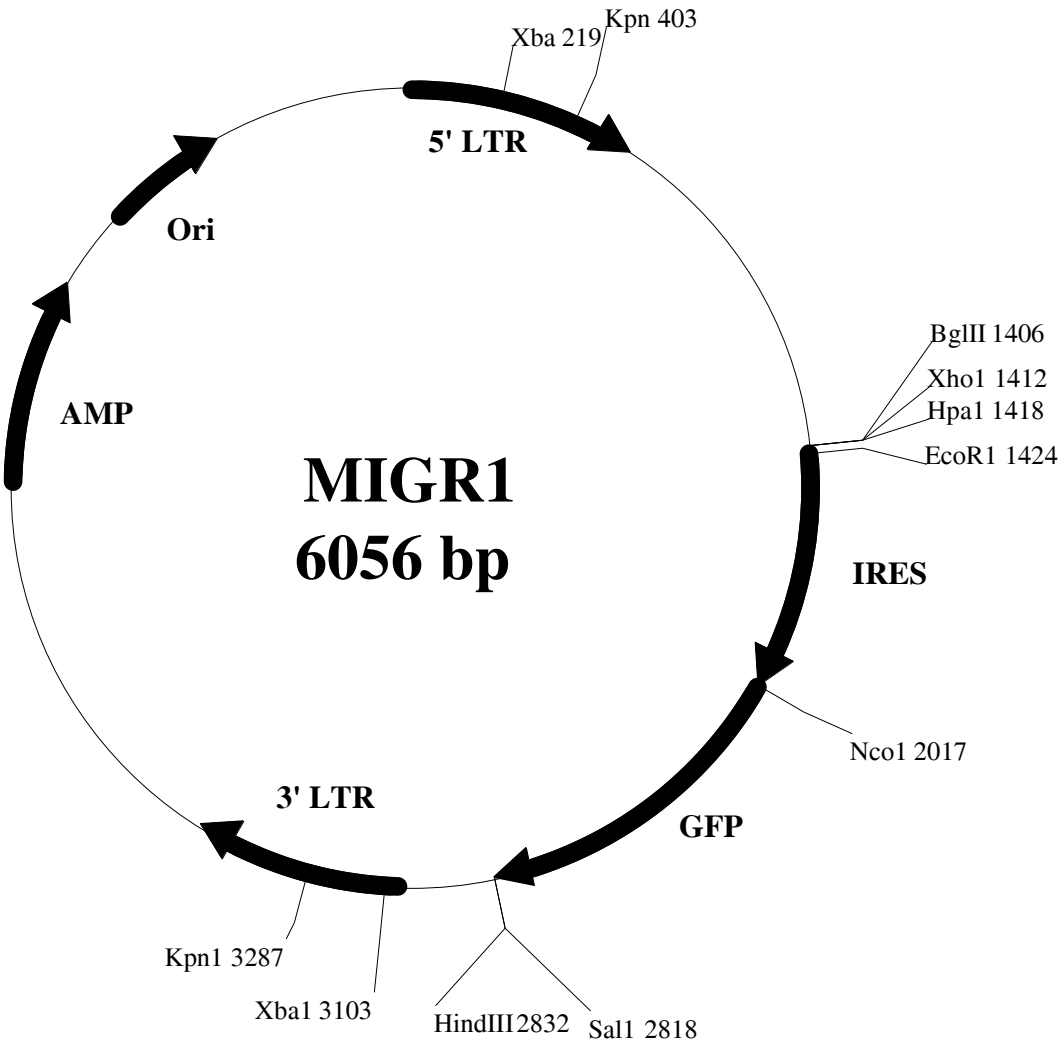


Figure C3: Vector map of MIGR1

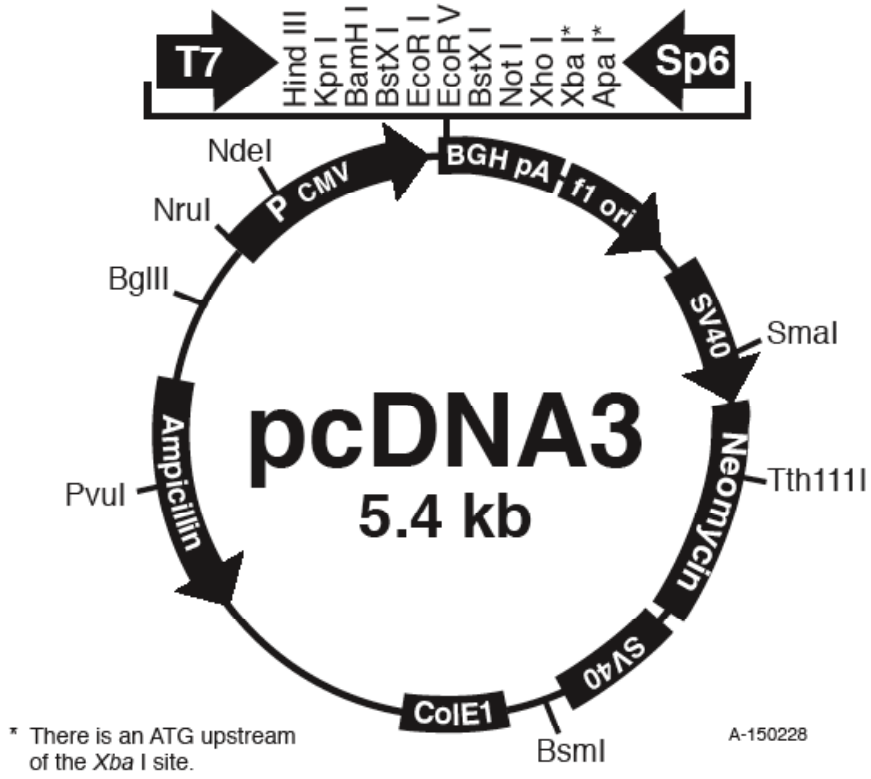


Figure C4: Vector map of pcDNA3

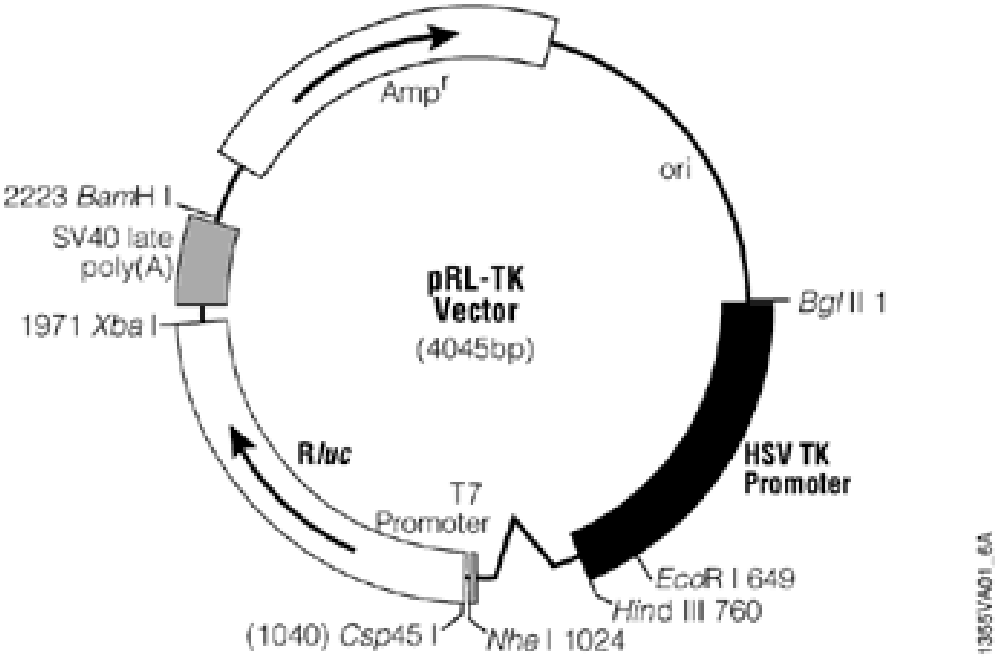


Figure C5: Vector map of pRL-TK

APPENDIX D

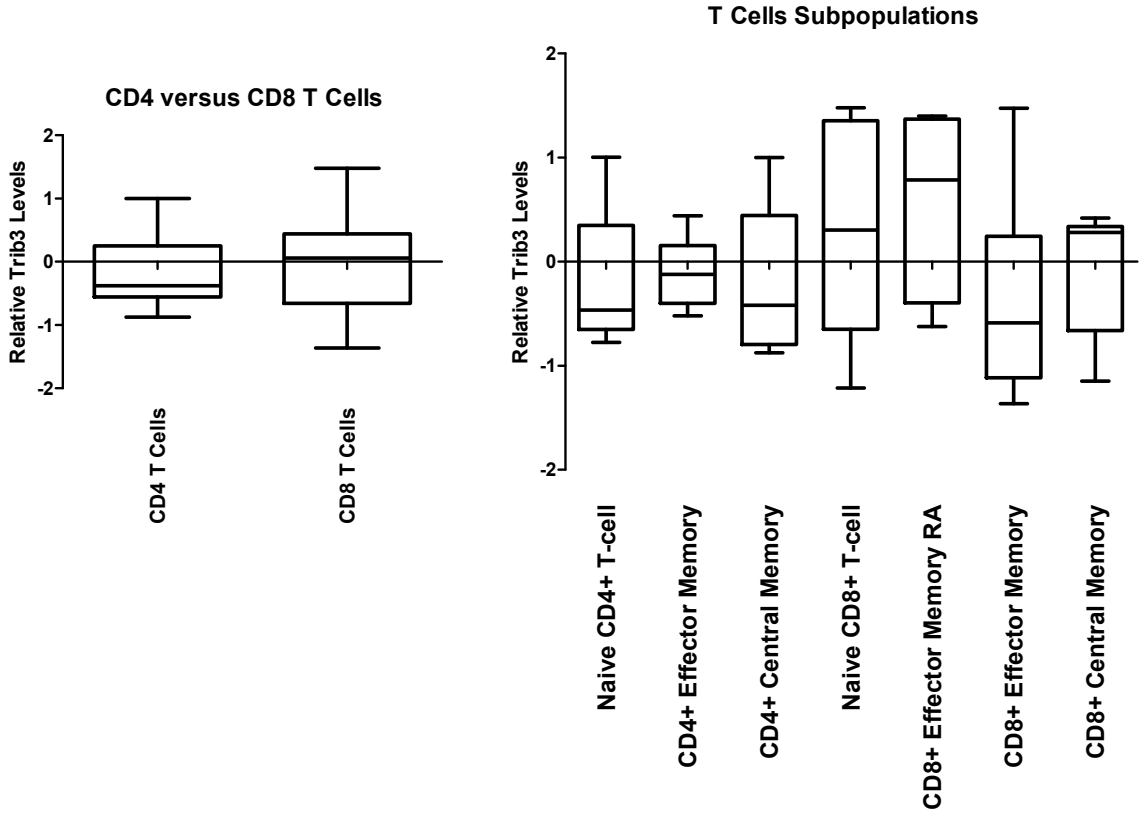


Figure D1: Expression profiles of TRIB3 in T cell subpopulations. Samples are plotted as a boxes and whiskers graph. The box extends from the 25th to 75th percentiles and the line in the boxes represents the median value of the samples. The whiskers extend from the maximum to the minimum sample value. Statistical analyses were carried out by performing a one-way ANOVA analyses followed by a Bonferroni's Multiple Comparison Test of each of the cell types versus the others in graphs with more than two plots using GraphPad Prism 5. In those with two plots the student t-test was performed instead. A p-value below 0.05 indicated a significant difference in gene expression the cell types, p-value below 0.05 is represented by *, below 0.01 by ** and below 0.001 by ***. CD4 T Cells, N = 21; CD8 T Cells, N = 24; Naive CD4+ T-cell, N = 7; CD4+ Effector Memory, N = 7; CD4+ Central Memory, N = 7; Naive CD8+ T-cell, N = 7; CD8+ Effector Memory RA, N = 4; CD8+ Effector Memory, N = 6; CD8+ Central Memory, N = 7.

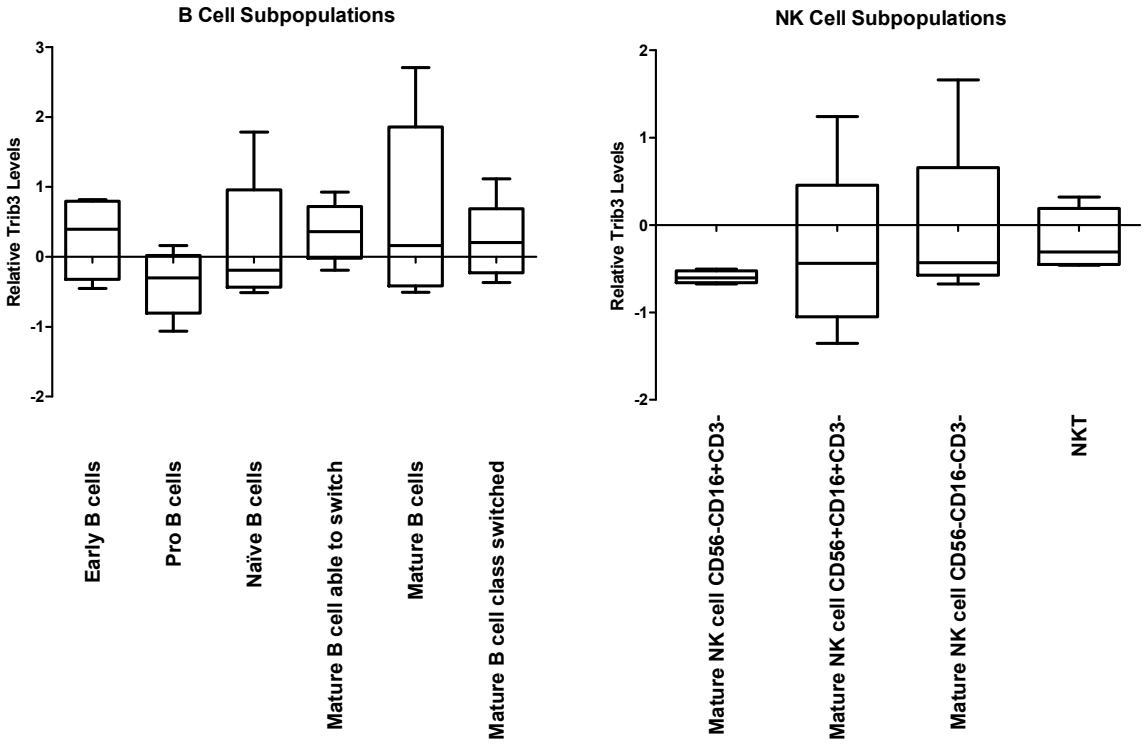


Figure D2: Expression profiles of TRIB3 expression in NK and B cell subpopulations.. Samples are plotted as a boxes and whiskers graph. The box extends from the 25th to 75th percentiles and the line in the boxes represents the median value of the samples. The whiskers extend from the maximum to the minimum sample value. Statistical analyses were carried out by performing a one-way ANOVA analyses followed by a Bonferroni's Multiple Comparison Test of each of the cell types versus the others in graphs with more than two plots using GraphPad Prism 5. In those with two plots the student t-test was performed instead. A p-value below 0.05 indicated a significant difference in gene expression the cell types, p-value below 0.05 is represented by *, below 0.01 by ** and below 0.001 by ***. Early B cells, N = 4; Pro B cells, N = 5; Naïve B cells, N = 5; Mature B cell able to switch, N = 5; Mature B cells, N = 5; Mature B cell class switched, N = 5; Mature NK cell CD56-CD16+CD3-, N = 4; Mature NK cell CD56+CD16+CD3-, N = 5; Mature NK cell CD56-CD16-CD3-, N = 5; NKT, N = 4.

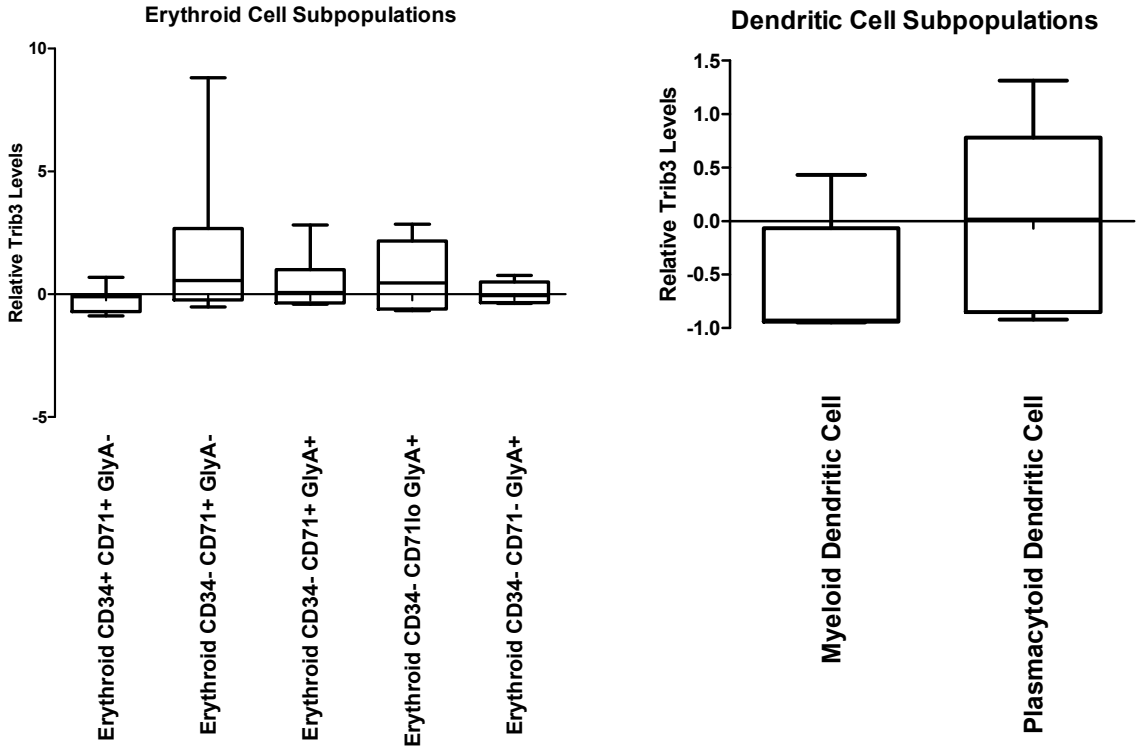
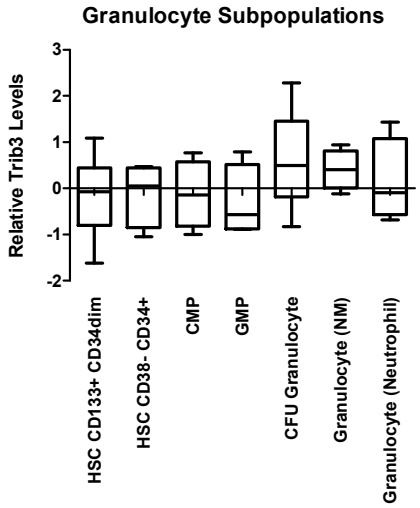
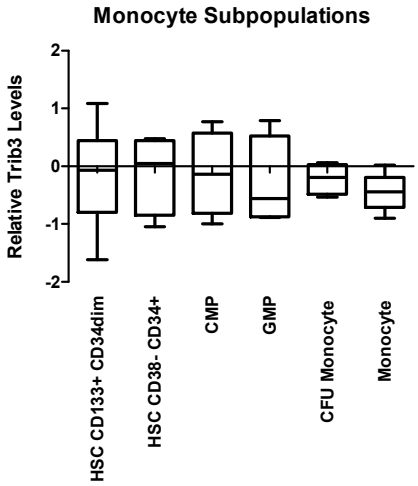
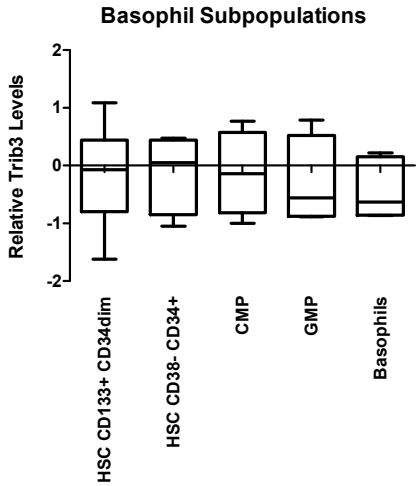
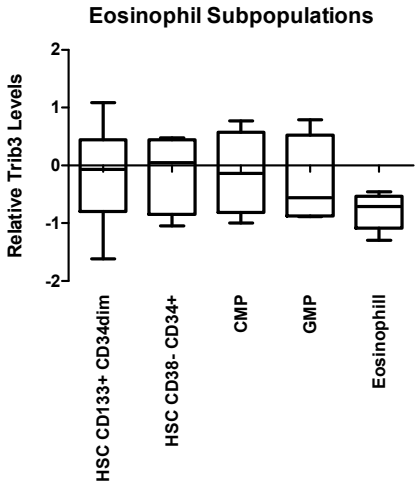


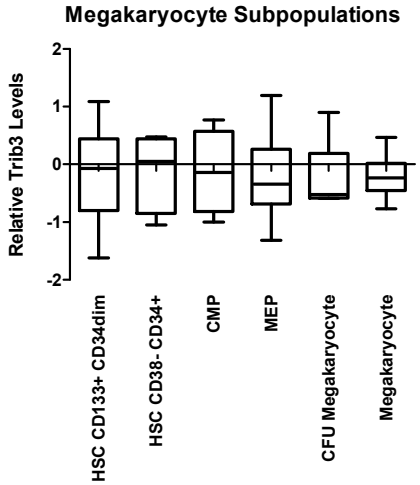
Figure D3: Expression profiles of TRIB3 in erythroid and dendritic cell subpopulations. Samples are plotted as a boxes and whiskers graph. The box extends from the 25th to 75th percentiles and the line in the boxes represents the median value of the samples. The whiskers extend from the maximum to the minimum sample value. Statistical analyses were carried out by performing a one-way ANOVA analyses followed by a Bonferroni's Multiple Comparison Test of each of the cell types versus the others in graphs with more than two plots using GraphPad Prism 5. In those with two plots the student t-test was performed instead. A p-value below 0.05 indicated a significant difference in gene expression the cell types, p-value below 0.05 is represented by *, below 0.01 by ** and below 0.001 by ***. Erythroid CD34+ CD71+ GlyA-, N = 7; Erythroid CD34- CD71+ GlyA-, N = 7; Erythroid CD34- CD71+ GlyA+, N = 6; Erythroid CD34- CD71lo GlyA+, N = 7; Erythroid CD34- CD71- GlyA+, N = 6; Myeloid Dendritic Cell, N = 5; Plasmacytoid Dendritic Cell, N = 5.


Granulocyte Subpopulations
Relative Trib3 Levels
3
2
1
0
-1
-2
HSC CD133+ CD34dim
HSC CD38- CD34+
CMP
GMP
CFU Granulocyte
Granulocyte (NM)
Granulocyte (Neutrophil)


Monocyte Subpopulations
Relative Trib3 Levels
2
1
0
-1
-2
HSC CD133+ CD34dim
HSC CD38- CD34+
CMP
GMP
CFU Monocyte
Monocyte


Basophil Subpopulations
Relative Trib3 Levels
2
1
0
-1
-2
HSC CD133+ CD34dim
HSC CD38- CD34+
CMP
GMP
Basophils


Eosinophil Subpopulations
Relative Trib3 Levels
2
1
0
-1
-2
HSC CD133+ CD34dim
HSC CD38- CD34+
CMP
GMP
Eosinophill


Megakaryocyte Subpopulations
Relative Trib3 Levels
2
1
0
-1
-2
HSC CD133+ CD34dim
HSC CD38- CD34+
CMP
MEP
CFU Megakaryocyte
Megakaryocyte

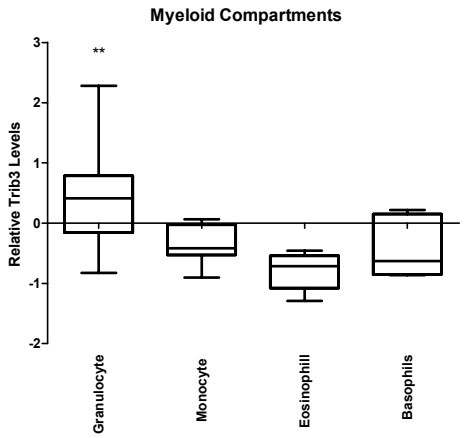

Myeloid Compartments
**
Relative Trib3 Levels
3
2
1
0
-1
-2
Granulocyte
Monocyte
Eosinophill
Basophils

Figure D4: Expression profiles of TRIB3 in granulocytes, monocytes, basophils, eosinophils, and megakaryocyte cellular subpopulations. Samples are plotted as a boxes and whiskers graph. The box extends from the 25th to 75th percentiles and the line in the boxes represents the median value of the samples. The whiskers extend from the maximum to the minimum sample value. Statistical analyses was carried out by performing a one-way ANOVA analyses followed by a Bonferroni's Multiple Comparison Test of each of the cell types versus the others using GraphPad Prism 5. A p-value below 0.05 indicated a significant difference in gene expression the cell types, p-value below 0.05 is represented by *, below 0.01 by ** and below 0.001 by ***. HSC CD133+ CD34dim, N = 10; HSC CD38- CD34+, N = 4; CMP, N = 4; GMP, N = 4; CFU Granulocyte, N = 5; Granulocyte (NM), N = 4; Granulocyte (Neutrophil), N = 4; CFU Monocyte, N = 4; Monocyte, N = 5; Basophils, N = 6; Eosinophill, N = 5; MEP, N = 9; CFU Megakaryocyte, N = 5; Megakaryocyte, N = 7; Granulocytes, N = 13; Monocytes, N = 9.

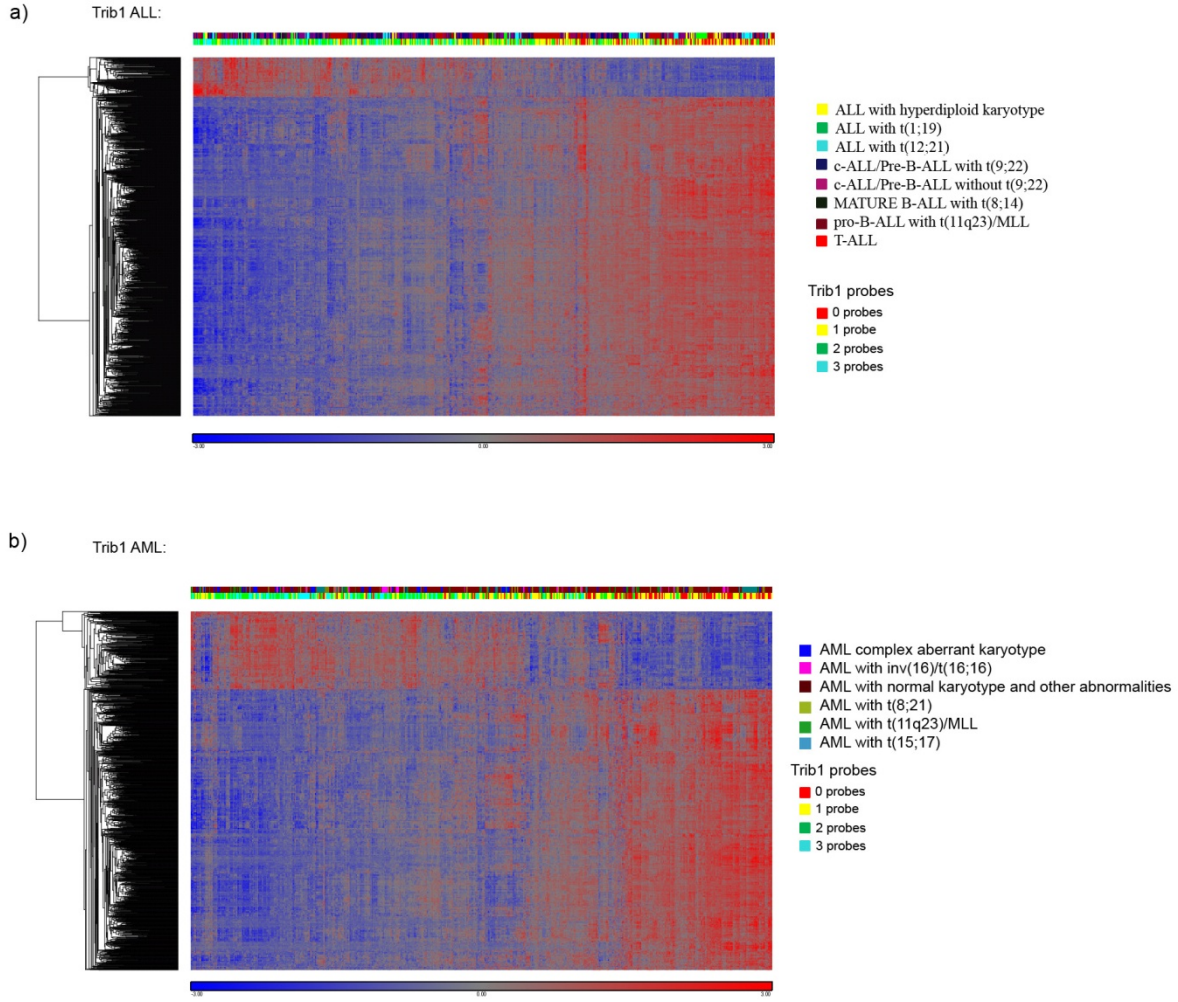


Figure D5: Heatmaps of TRIB1 and its gene neighbours clustered based on TRIB1 expression in the a) ALL and b) AML patient samples of the MILE study. To determine the nearest-neighbours of TRIB1 in the MILE dataset for ALL and AML each patient sample was separated based on whether TRIB1 expression was below the median expression of each of the three probe for TRIB1 in the microarray (labelled 0) or above the median expression in one (labelled 1), two (labelled 2) or three (labelled 3) of the probes sets. A one-way ANOVA analysis was then performed to determine genes with significantly different expression between the 0 and 3 group (below versus above the median for all three probe sets). Unsupervised hierarchical clustering of the top differentially expressed genes was then performed using PARTEK GENOMICS SUITE (Version 6.6) for the ALL samples, AML samples. See supplementary table 3.I for gene neighbours lists used to generate heatmaps on the accompanying CD.

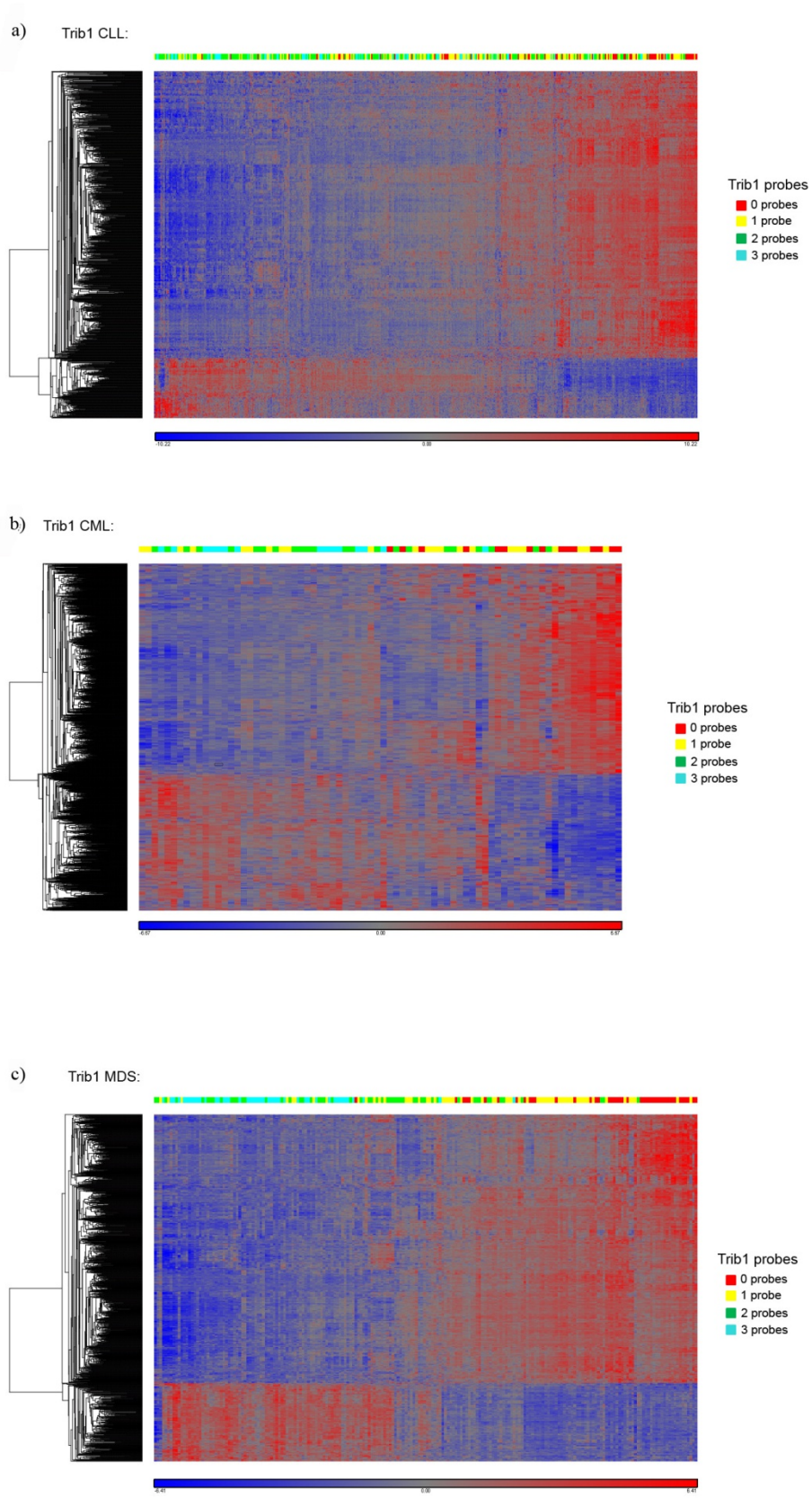


Figure D6: Heatmaps of TRIB1 and its gene neighbours clustered based on TRIB1 expression in the a) CLL, b) CML and c) MDS patient samples of the MILE study. To determine the nearest-neighbours of TRIB1 in the MILE dataset for CLL, CML

and MDS, each patient sample was separated based on whether TRIB1 expression was below the median expression of each of the three probe for TRIB1 in the microarray (labelled 0) or above the median expression in one (labelled 1), two (labelled 2) or three (labelled 3) of the probes sets. A one-way ANOVA analysis was then performed to determine genes with significantly different expression between the 0 and 3 group (below versus above the median for all three probe sets). Unsupervised hierarchical clustering of the top differentially expressed genes was then performed using PARTEK GENOMICS SUITE (Version 6.6) for the CLL samples, CML samples and MDS samples. See supplementary table 3.I for gene neighbours lists used to generate heatmaps on the accompanying CD.

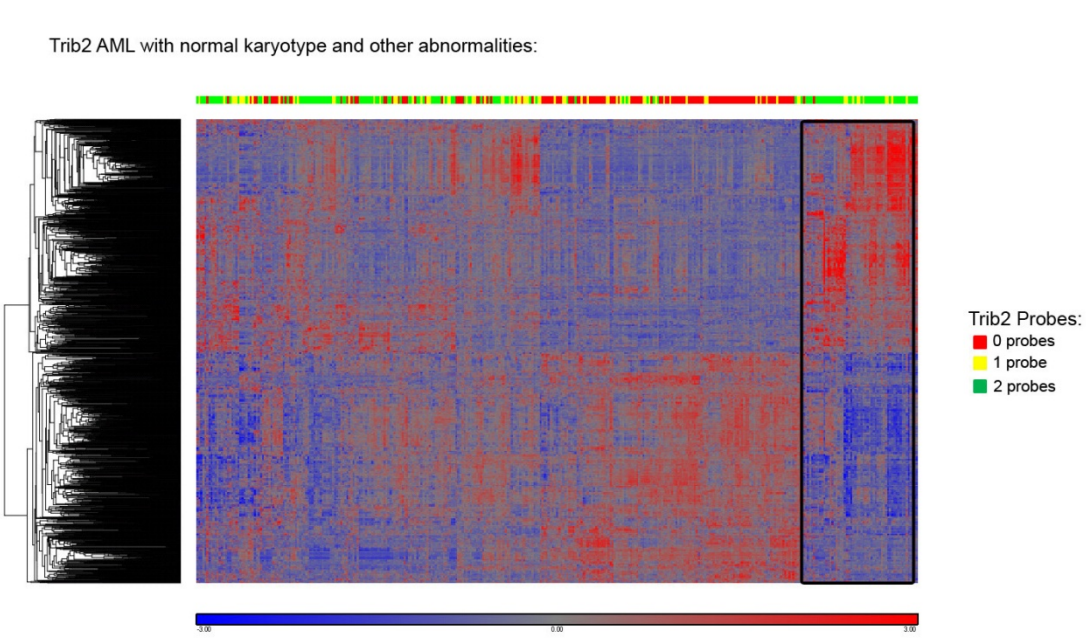


Figure D7: Heatmaps of TRIB2 and its gene neighbours clustered based on TRIB2 expression in the AML with normal karyotype and other abnormalities patient samples of the MILE study. To determine the nearest-neighbours of TRIB2 in the MILE dataset each patient sample was separated based on whether TRIB2 expression was below the median expression of each of the three probe for TRIB2 in the microarray (labelled 0) or above the median expression in one (labelled 1), two (labelled 2) or three (labelled 3) of the probes sets. A one-way ANOVA analysis was then performed to determine genes with significantly different expression between the 0 and 3 group (below versus above the median for all three probe sets). Unsupervised hierarchical clustering of the top differentially expressed genes was then performed using PARTEK GENOMICS SUITE (Version 6.6). See supplementary table 3.II for gene neighbours list used to generate the heatmap on the accompanying CD.

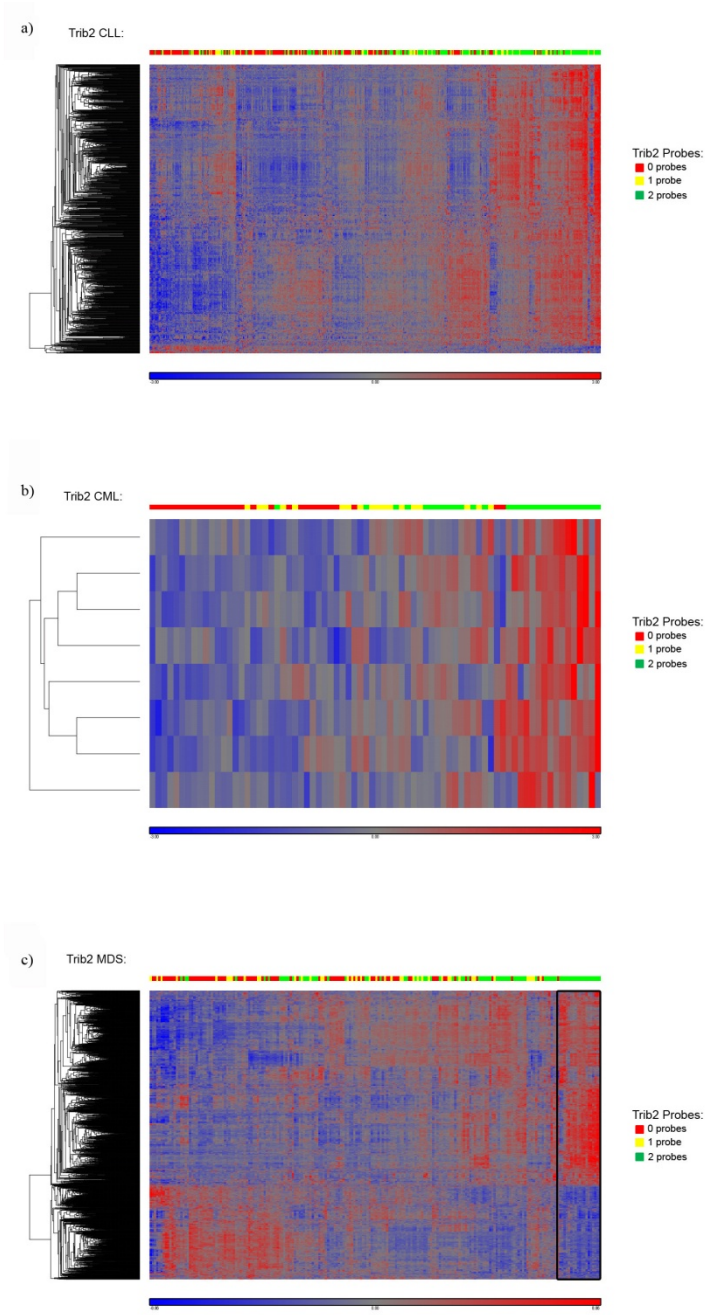


Figure D8: Heatmaps of TRIB2 and its gene neighbours clustered based on TRIB2 expression in the a) CLL, b) CML and c) MDS patient samples of the MILE study. To determine the nearest-neighbours of TRIB2 in the MILE dataset each patient sample was separated based on whether TRIB2 expression was below the median expression of each of the three probe for TRIB2 in the microarray (labelled 0) or above the median expression in one (labelled 1), two (labelled 2) or three (labelled 3) of the probes sets. A one-way ANOVA analysis was then performed to determine genes with significantly different expression between the 0 and 3 group (below versus above the median for all three probe sets). Unsupervised hierarchical clustering of the top differentially expressed genes was then performed using PARTEK GENOMICS SUITE (Version 6.6). See supplementary table 3.II for gene neighbours lists used to generate heatmaps on the accompanying CD.

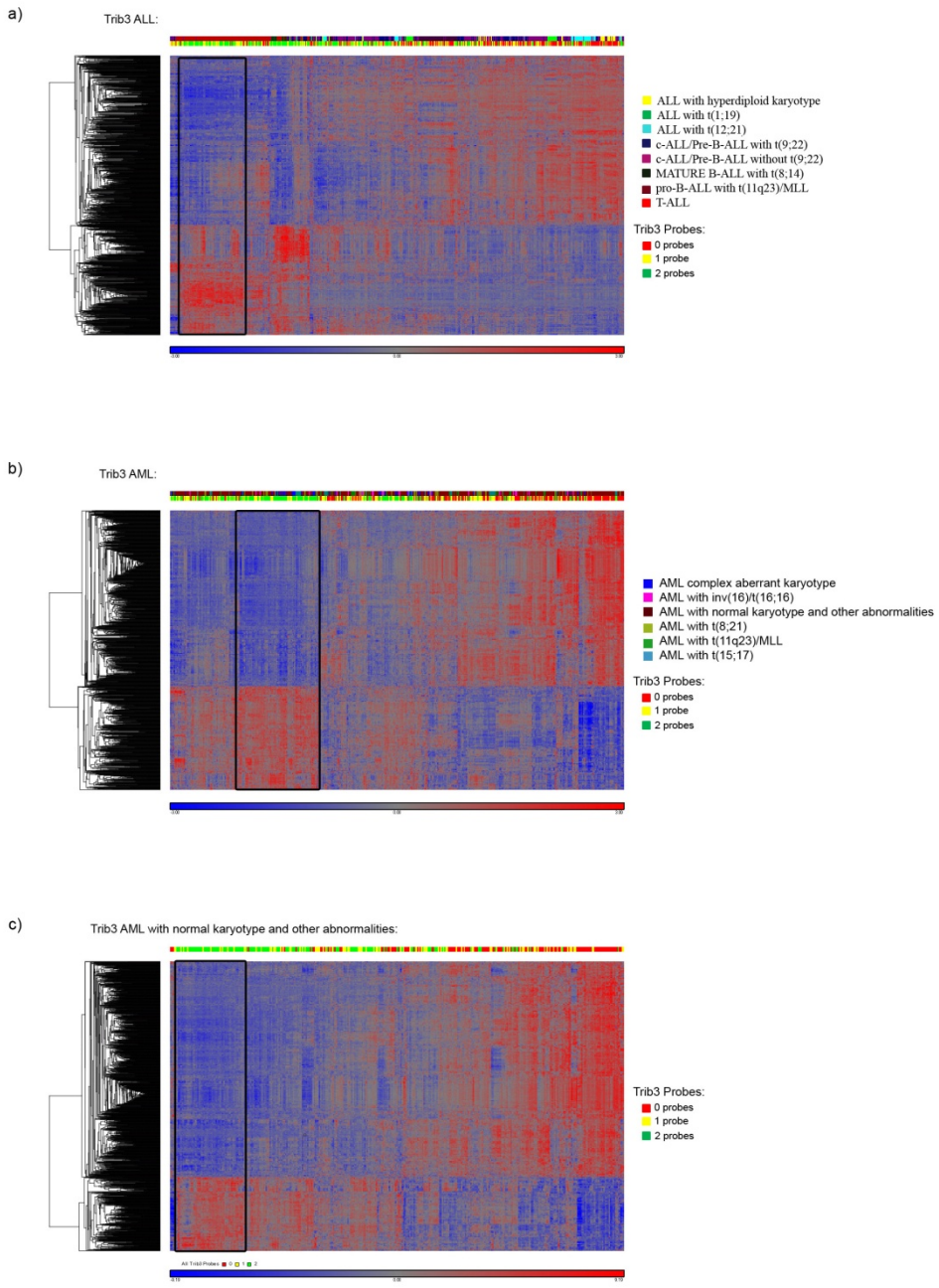


Figure D9: Heatmaps of TRIB3 and its gene neighbours clustered based on TRIB3 expression in the a) ALL, b) AML and c) AML with normal karyotype and other abnormalities patient samples of the MILE study. To determine the nearest-neighbours of TRIB1 in the MILE dataset for ALL, AML or AML with normal karyotype and other abnormalities each patient sample was separated based on whether TRIB1 expression was below the median expression of each of the three probe for TRIB1 in the microarray (labelled 0) or above the median expression in one (labelled 1), two (labelled 2) or three (labelled 3) of the probes sets. A one-way ANOVA analysis was then performed to determine genes with significantly different expression between the 0 and 3 group (below versus above the median for all three probe sets). Unsupervised hierarchical clustering of the top differentially expressed genes was then performed using PARTEK GENOMICS SUITE (Version 6.6). See

supplementary table 3.III for gene neighbours lists used to generate heatmaps on the accompanying CD.

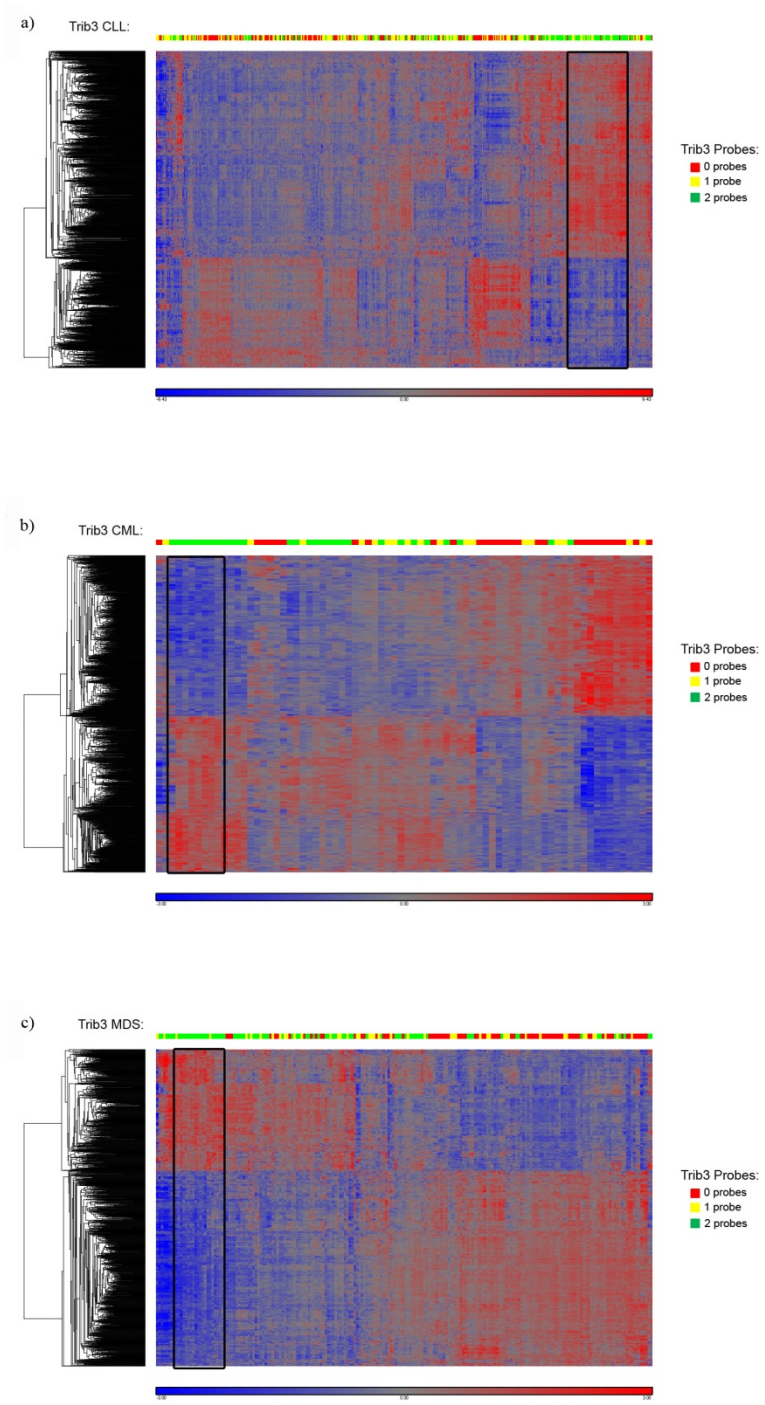


Figure D10: Heatmaps of TRIB3 and its gene neighbours clustered based on TRIB3 expression in the a) CLL, b) CML and c) MDS patient samples of the MILE study. To determine the nearest-neighbours of TRIB1 in the MILE dataset for CLL, CML or MDS each patient sample was separated based on whether TRIB1 expression was below the median expression of each of the three probe for TRIB1 in the microarray (labelled 0) or above the median expression in one (labelled 1), two (labelled 2) or three (labelled 3) of the probes sets. A one-way ANOVA analysis was then performed to determine genes with significantly different expression between the 0 and 3 group (below versus above the median for all three probe sets). Unsupervised hierarchical clustering of the top differentially expressed genes was then performed

using PARTEK GENOMICS SUITE (Version 6.6). See supplementary table 3.III for gene neighbours lists used to generate heatmaps on the accompanying CD.

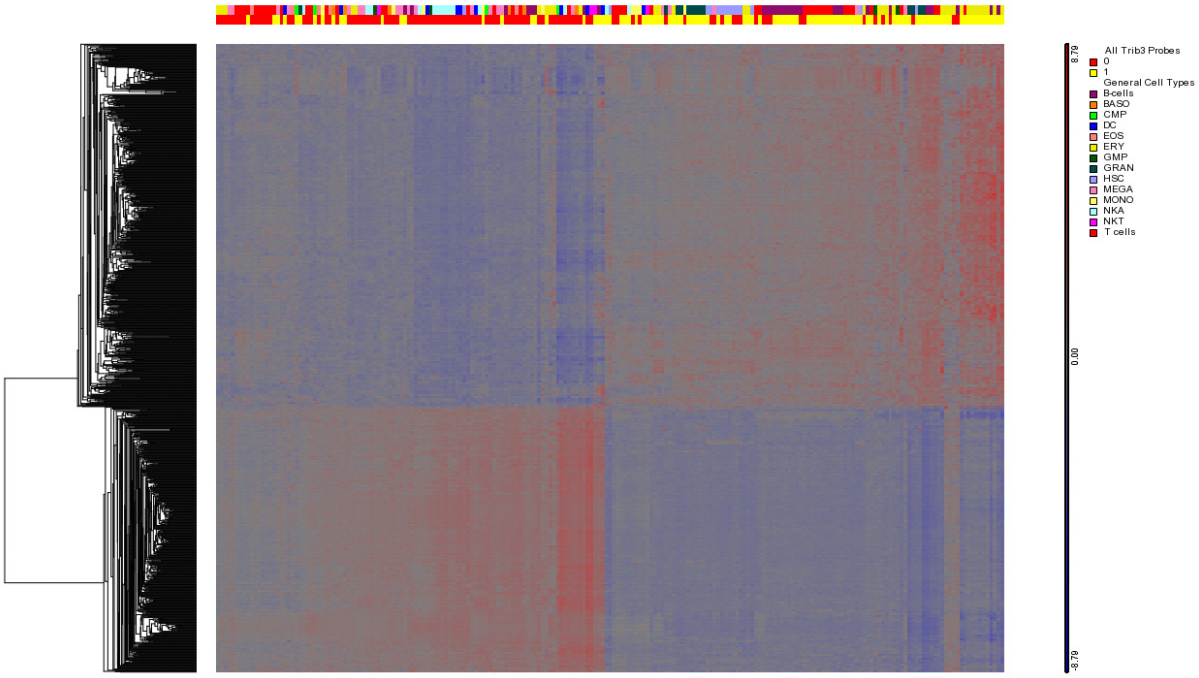


Figure D11: Heatmap of TRIB3 gene neighbours clustered based on TRIB3 expression in the cells of the haematopoietic system. To determine the nearest-neighbours of TRIB3 each sample was separated based on whether TRIB3 expression was below the median expression (labelled 0) or above the median expression (labelled 1) in its relevant probe. A one-way ANOVA analysis was then performed to determine genes with significantly different expression between the 0 and 1 group (below versus above the median for the probe sets). Unsupervised hierarchical clustering of the top 1063 differentially expressed genes with a p-value of 0.000000000231048 or less as determined by the ANOVA analysis was then performed using PARTEK GENOMICS SUITE (Version 6.6). See supplementary table 3.IV for gene neighbours lists used to generate heatmaps on the accompanying CD.

ALL							
rank	cmap name	mean	n	enrichment	p	specificity	percent non-null
1	isometheptene	0.636	4	0.922	0.00004	<0.00001	100
2	doxylamine	-0.549	5	-0.85	0.00024	0.016	100
3	clioquinol	0.6	5	0.819	0.00042	0.0209	100
4	STOCK1N-35215	0.688	3	0.936	0.00044	<0.00001	100
5	prochlorperazine	0.304	16	0.483	0.00044	0.1602	62
6	salsolidin	-0.569	4	-0.876	0.00052	<0.00001	100
7	penbutolol	0.686	3	0.929	0.00064	<0.00001	100
8	rescinnamine	0.621	3	0.925	0.0008	0.0052	100
9	arachidonic acid	-0.642	3	-0.918	0.00096	<0.00001	100
10	dosulepin	0.612	4	0.838	0.00107	0.0154	100
AML							
rank	cmap name	mean	n	enrichment	p	specificity	percent non-null
1	LY-294002	-0.268	61	-0.36	<0.00001	0.2331	52
2	Prestwick-857	0.557	4	0.864	0.00046	0.0065	100
3	0198306-0000	-0.587	4	-0.88	0.00052	<0.00001	100
4	atractyloside	0.564	5	0.816	0.00052	0.0111	100
5	mometasone	0.623	4	0.851	0.00072	0.0103	100
6	helveticoside	0.481	6	0.743	0.00077	0.0851	100
7	5230742	-0.708	2	-0.978	0.00107	<0.00001	100
8	pargyline	-0.556	4	-0.839	0.00119	<0.00001	100
9	indoprofen	0.483	4	0.827	0.00139	0.0124	100
10	cefalexin	-0.447	5	-0.762	0.00144	<0.00001	80
MDS							
rank	cmap name	mean	n	enrichment	p	specificity	percent non-null
1	helveticoside	0.657	6	0.819	0.00008	0.0255	100
2	meptazinol	-0.691	4	-0.877	0.00052	<0.00001	100
3	monensin	0.419	6	0.75	0.00062	0.0318	83
4	levomepromazine	0.583	4	0.85	0.00072	0.0195	100
5	lycorine	0.555	5	0.774	0.0013	0.037	100
6	latamoxef	-0.59	3	-0.893	0.00234	0.0058	100
7	valinomycin	0.508	4	0.812	0.00237	0.0349	100
8	digoxigenin	0.495	5	0.744	0.0025	0.0614	80
9	15-delta prostaglandin J2	0.321	15	0.446	0.00284	0.3296	66
10	CP-645525-01	0.54	3	0.884	0.00314	0.0056	100
CLL							
rank	cmap name	mean	n	enrichment	p	specificity	percent non-null
1	LY-294002	-0.307	61	-0.368	<0.00001	0.2209	50
2	calcium pantothenate	-0.822	4	-0.908	0.0001	0	100
3	sirolimus	-0.286	44	-0.314	0.00028	0.2945	50
4	etynodiol	0.832	4	0.871	0.00036	0	100
5	F0447-0125	0.818	4	0.862	0.0005	0	100
6	acetylsalicylic acid	-0.475	13	-0.535	0.00076	0	76
7	Gly-His-Lys	0.822	3	0.919	0.00114	0	100
8	ronidazole	-0.765	3	-0.91	0.00128	0	100
9	mometasone	0.722	4	0.826	0.00145	0.0103	100
10	cetirizine	-0.795	4	-0.815	0.00215	0	100
CML							
rank	cmap name	mean	n	enrichment	p	specificity	percent non-null
1	LY-294002	-0.407	61	-0.467	<0.00001	0.0675	63
2	chloroquine	0.586	4	0.888	0.00016	0	100
3	wortmannin	-0.389	18	-0.495	0.0002	0.1147	61
4	STOCK1N-35215	0.658	3	0.941	0.00026	0	100
5	hexylcaine	0.602	4	0.856	0.00056	0	100
6	pirenperone	0.329	5	0.812	0.00058	0	60
7	mefloquine	-0.443	5	-0.775	0.00098	0.0278	80
8	chlorphenesin	0.567	4	0.827	0.00135	0	100
9	fenofibrate	0.614	3	0.913	0.00138	0	100
10	F0447-0125	0.467	4	0.816	0.00211	0	75

Table DI: Top 10 cmap small molecules enriched for the TRIB3 gene pattern in ALL, AML, MDS, CLL and CML. Complete list of enrichment results as well as break down of results based on cmap name and cell line or by atc code can be found in supplementary tables 3.XVIII to 3.XXII on the accompanying CD. TRIB3 signatures used to run these analyses can be found in supplementary table 3.XXIII on the accompanying CD.